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CLINICAL LABORATORY METHODS AND DIAGNOSIS

VOLUME II

CLINICAL LABORATORY METHODS AND DIAGNOSIS

A Textbook on Laboratory Procedures With Their Interpretation

By

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CHAPTER VII

EXAMINATION OF PUNCTURE FLUIDS

GENERAL CONSIDERATIONS

Puncture fluids should be obtained under all sterile precautions. The skin of the patient should be iodized and afterward rubbed with alcohol. The hands of the operator should be covered with sterile rubber gloves. The puncture instrument should be sterilized by dry heat at 170° C. for two hours.

1. **Amount** of fluid withdrawn should be noted. In cases of fluid accumulation in the pleural cavities, as much as 1.5 to 2 liters can be obtained; in abdominal ascites, as much as 10 to 20 liters.

2. **Gross Appearance.**—Note whether it is clear or cloudy; serous or chylous or purulent; colorless or colored; whether blood or bile stained. The presence of small pieces of blood clots does not change the appearance much. At times these small clots may be recognized after centrifugalization. It is only when hemolysis has taken place that the fluid takes on a red or yellowish color. In cases of biliary stasis, the fluid may be colored yellow. Where blood admixtures are present, eliminating the question of trauma, tuberculosis, or carcinoma of the serous surfaces must be considered.

3. A fetid **odor** is, of course, indicative of a severe inflammatory purulent infection.

4. **Specific Gravity** may be estimated by means of an ordinary urinometer. This investigation should be undertaken at once before precipitation of fibrin may alter the results. The specific gravity will enable one at times to differentiate between a **transudate** and an **exudate,** the borderline between the two being 1.018. Fluids with a low specific gravity are suggestive of a transudate (usually due to obstructive changes in circulatory apparatus), while higher specific gravities speak for exudates due to inflammatory processes. Practically all exudates are rich in fibrinogen, whereas transudates are usually poor in fibrinogen. When these exudates settle, the fibrin clots usually carry down with them some cellular elements.

5. **Albumin Content.**—Transudates are transparent, light yellow or light green; exudates are serous, bloody, purulent, or gangrenous. They also differ materially in their albumin content. Transudates contain from 1 to 3 per cent, or less, albumin, while exudates contain from 4 to 6 per cent, or more.

The quantity of albumin may be estimated by means of the Esbach method. (See Esbach Method in Urine Analysis.) It is best to dilute these fluids before the test, so that the albumin content will not be higher than 10 per cent. In other words, dilute them five to ten times, afterward mathematically correcting for the dilution. It is best to make the dilution with albumin-free urine instead of with water.

6. **The Rivalta Test.**—The Rivalta test is a simple qualitative determination of the globulin fraction of albumin present.

Add a few drops of 0.5% watery acetic acid solution by means of a pipette to a test tube containing the fluid to be examined. Add these drops slowly, one by one.

In fluids with a rich amount of globulin the precipitate appears in a form simulating cigarette smoke. A bluish cloudiness, which slowly falls to the bottom, develops in exudates; in transudates, where the globulin content is extremely minute, the cloudiness remains suspended in the fluid.

7. Determination of **bile** present may be carried out just as in urine, page 72.

8. **Microscopic Examination.**—The microscopic examination should be made very soon after the fluid is taken from the body before any clotting or precipitation occurs. If it is not possible to do this at once, it is best to add a small amount of 5 to 10 per cent sodium citrate or 4 per cent ammonium oxalate solution or a few crystals of hirudin to prevent clotting.

The nature of the cells present is very important. It is best to first prepare a smear direct from the fluid and stain just like a blood film. In tuberculous infections a great many lymphocytes are noted. The presence of neutrophilic leukocytes speaks of purulent or infectious processes. The presence of eosinophiles is of no particular importance except, possibly, as an allergic phenomenon.

Bacteriologic Investigations.—For bacteriologic investigations, refer to Chapter X.

CELLULAR STRUCTURE OF EFFUSIONS

The study of the cellular structure of effusions has been a neglected laboratory procedure. Possibly the most satisfactory method of study is that advised by Mandlebaum,[1] and popularized by Goldman.[2]

Mandlebaum Method.—

Place the fluid in a large Erlenmeyer flask. Allow to stand overnight in a refrigerator.

Decant the supernatant fluid.

Pour the sediment into a 50 c.c. centrifuge tube.

Centrifuge 20 minutes at moderate speed.

Again decant the supernatant fluid.

Harden the sediment with 1:10 Formalin or Zenker's fluid for 24 hours.

Carry through dehydrating and embedding steps as an ordinary tissue. Cut from above down so as to include all the cellular elements. Stain in hematoxylin-eosin.

Helwig Method.[3]—

Add enough Formalin immediately to the specimen to make a 4% solution of the fixative. This prevents bacterial growth and also cell degeneration.

---

[1] Mandlebaum, F. S.: Proc. New York Path. Soc., 1900, p. 178.
[2] Goldman, Alfred: Arch. Surg. 19: 1672, 1929.
[3] Helwig, E. B.: J. Missouri M. A. 39: 73, 1942.

Centrifuge as soon as possible.

There is a tendency for the block of sediment to crumble if sectioned immediately after fixation by formaldehyde; the entire mass of sediment is cut in a vertical plane when paraffin sections are prepared.

For further information on the preparation of stained sections of puncture fluids, see section on tissue cutting and staining, page 2156.

Goldman made an interesting report of fifty effusions prepared by this method from serous fluids in cases of malignant, cardiac, and tuberculous disease. The specific gravity was noted in all fluids. There were twenty cases of malignant disease in this series proved by autopsy, biopsy, or a palpable mass with gross characteristics of a malignant tumor. He studied the effusions from all these cases with particular reference to the presence or absence of tumor cells. Zemansky, in his series studied by this method, showed that in 60 per cent of fifty-five cases of carcinoma there were tumor cells in the effusions. In Goldman's report, 80 per cent of the effusions showed cells which were interpreted as being malignant. In other words, a positive diagnosis of tumor cells may be made with the highest degree of accuracy. A negative diagnosis does not, of course, rule out a malignant condition. Obviously, there must be contact between the tumor and the fluid before tumor cells are "shed."

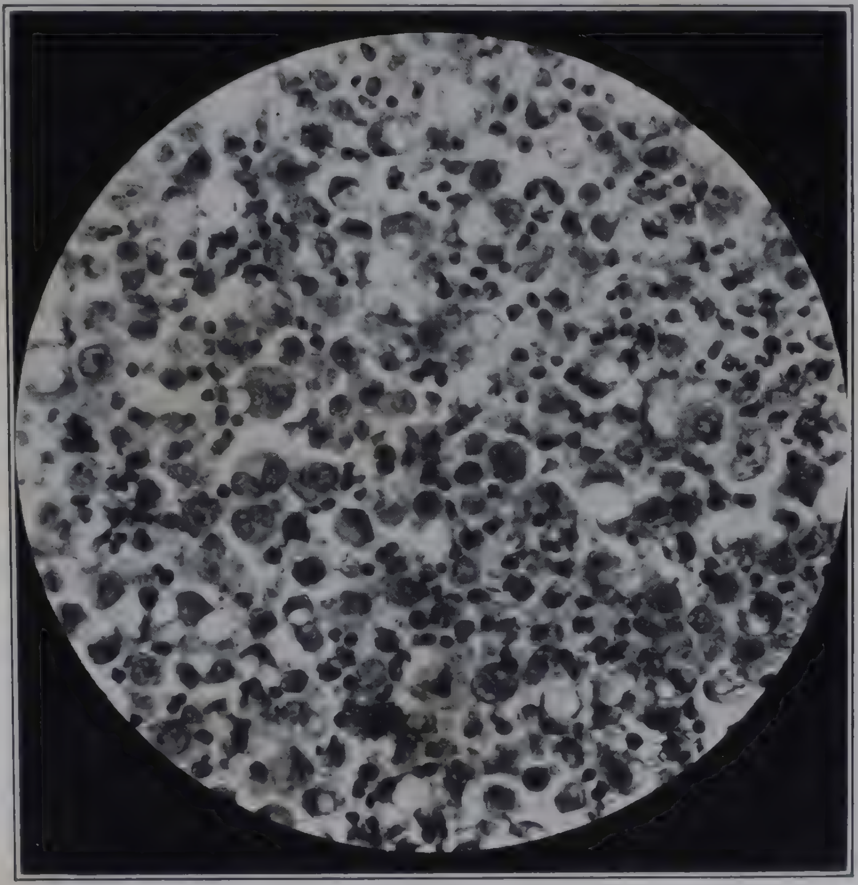

Fig. 296.—Paraffin section of pleural fluid showing carcinoma cells. (×400.)

Tumor cells, as has been pointed out by Zemansky and others, appear in two main forms: (a) fragments of tumor tissue often showing glandular or pseudoglandular arrangements or clumps of tumor cells, and (b) isolated tumor cells, the characteristics of which will be mentioned later.

The first type is easy to diagnose. The cell clumps stand out as large, deep-staining cells, amid various types of blood cells and mesothelial cells. Occasionally the tumor fragments may be seen almost exclusively at the bottom of the centrifuge tube.

It is in those cases in which individual tumor cells occur that one may have difficulty in making a diagnosis. There were three such cases in Goldman's series. Criteria for diagnosis here are large atypical deep-staining cells, with a large excentric nucleus or with several nuclei, sometimes showing mitotic figures, often vacuolated. Mesothelial cells which may or may not be clumped, occurring in transudates of long standing, are at times confusing. These must be carefully studied and differentiated by the criteria mentioned.

The cells often assume a glandular arrangement (eight cases). The tumor cells may thus resemble the original tumor. In one case both pleural and peritoneal fluids were obtained, and both specimens showed the same type of cell.

A fluid may be positive at one examination and later appear negative. A patient with carcinoma of the pleura in whom the condition had metastasized from a bronchial carcinoma developed an effusion which on the first examination revealed many tumor cells. Two months later, a second tapping was done in which no tumor cells were found. It is possible that the fluid obtained from the second tapping came from an encysted effusion and not from the large pleural effusion. The sputum in this case was treated by the same method and revealed no tumor cells.

Case 32 of Goldman's series shows the necessity of careful search for tumor cells. While in the majority of cases cells are present in abundance, in this instance only one group of cells was found on which the diagnosis of malignant disease was made. A biopsy of a tumor of the neck revealed a metastatic carcinoma probably from the bronchus.

Tumor cells may appear in an effusion soon after the appearance of the fluid.

Four cases in which a positive diagnosis of tumor cells was made came to autopsy. All showed direct contact of cancer tissue with the effusions which had developed. Apparently this is essential before tumor cells are "shed" into the serous cavity.

Four negative examinations occurred in proved cases of carcinoma. They were negative because there was no actual contact of tumor and fluid. Goldman states: "The appearance of the effusions varied from a clear straw-colored fluid as seen in transudates to turbid, bloody fluids. All specimens contained red blood cells in large amounts, more than in any other type of effusion. The specific gravity of the fluids ranged from 1.013 to 1.025; the average was 1.017. This figure is appreciably lower than that usually given in textbooks. There was no apparent relation between the specific gravity and the number of tumor cells present."

With respect to cardiac effusions, all contained numerous red blood cells, less than in the cases of tumor and more than in the cases of tuberculosis.

The predominating cell was the mesothelial cell; there were also a few macrophages containing blood pigment, similar to the "heart failure" cell in the sputum in cardiac cases. There were a few lymphocytes and occasional polymorphonuclear neutrophiles. Goldman warns against mistaking clumped mesothelial cells for tumor cells. The former are smaller, more regular, have a single nucleus, show no mitosis, and do not stain so deeply as do the tumor cells. The specific gravity of these fluids varied from 1.008 to 1.014, with an average of 1.012.

Tuberculous effusions showed lymphocytes as the predominating cell. In some cases, polymorphonuclear cells predominated. A few mononuclears were usually present; also a few red cells. Specific gravity ranged from 1.019 to 1.026. Goldman studied a number of miscellaneous cases with effusions. A pleural effusion in a case of Hodgkin's disease showed an equal mixture of lymphocytes and large mononuclear cells.

It is in carcinoma of the pleura that we have the greatest success in proving the diagnosis during life by this method.

One of the very important considerations in examination of fluids for carcinoma is that the carcinomatous cells show a very fine arrangement of mitotic figures. Fatty degeneration in the cells is likely to be found.

Helwig[1] reported on a study of sixty-five routine diagnostic fluids obtained from fifty-six patients. Paraffin sections were prepared and stained with hematoxylin and eosin. The sections were examined solely on a morphologic basis without knowledge of the history of the patient or of the cavity from which the fluid had been removed. Each specimen was recorded either as "positive for tumor cells" or as "negative for tumor cells." All the criteria which could be considered of possible significance for judging the presence or absence of tumor cells were listed on a chart. The criteria were recorded as plus or minus on each fluid. He utilized the following criteria: mitoses, abnormal mitoses, large nuclei, large or small nucleoli, multiple nuclei, position of the nucleus, irregularity of cell outline, vacuolization of the cytoplasm, acini, papillae, clumps of cells, fibrin, and red blood cells.

His results were as follows:

TABLE 132A.—SUMMARY OF SPECIMENS REPORTED POSITIVE

	PERITONEAL FLUID	PLEURAL FLUID	TOTAL
Total patients	23	33	56
Total specimens	29	36	65
Total reported positive	10	8	18
Proved correct[1]	4	2	6
Presumed correct[2]	3	6	9
Proved incorrect[1]	1	0	1
Unknown[4]	2	0	2

[1], [2], [4]See footnotes under Table 132B, p. 1226.

It will be noted that two cases showing the result "negative for tumor cells" were a primary carcinoma of the liver and the other a primary carcinoma

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[1] Helwig: Loc. cit.

TABLE 132B.—SUMMARY OF SPECIMENS REPORTED NEGATIVE

	PERITONEAL FLUID	PLEURAL FLUID	TOTAL
Total patients	23	33	56
Total specimens	29	36	65
Total reported negative	19	28	47
Proved correct[1]	9	6	15
Presumed correct[3]	5	10	15
Proved incorrect[1]	3	0	3
Presumed incorrect[2]	1	10	11
Unknown[4]	1	2	3

[1] Diagnosis established by examination of body cavity at operation or autopsy or by the identification of a specific etiologic agent in the cavity fluid.

[2] Diagnosis established by biopsy of malignant tumor in the patient or by roentgen-ray examination or by palpation of a mass when considered with the clinical course.

[3] Diagnosis established by the results of laboratory tests when considered with the clinical course.

[4] The exact nature of the disease was not established.

of the pancreas, the peritoneum being free from metastases at autopsy. Obviously, an examination of ascitic fluid from these two cases could not reveal the presence of a tumor which did not involve the peritoneum and the diagnosis of ''negative for tumor cells'' was actually correct for the specimen examined. He narrated that, in another case, a single examination of ascitic fluid failed to reveal the presence of identifiable tumor cells although an autopsy revealed a neoplasm involving the peritoneum.

His work emphasizes that in judging the accuracy of this type of test, it is necessary not only to know whether a neoplasm is present in the patient but also to know whether the serous surface of the particular body cavity from which the fluid was withdrawn is involved by the tumor. Even though a patient has a neoplasm, tumor cells cannot possibly be identified in fluid obtained from a cavity which is not involved by the neoplasm.

The criteria of Zemansky[1] in detail, which are useful in judging a fluid positive or not for neoplastic cells, are (1) fragments of tissue with a definite arrangement of cells and stroma, (2) multiple groups of large, deeply staining cells, giving the slide a mottled appearance, (3) finer cellular changes such as extreme irregularity of cell outline, excentricity of nucleus, extremely large size of nucleus, multinucleation, and typical or atypical mitotic figures. Foot,[2] some ten years after Zemansky's publication, attempted a diagnosis by means of measurement of the nucleolar-nuclear ratio. He refused to accept as evidence of neoplasia the presence of hyperchromasia, large and deeply stained nuclei, multinucleation, mitotic figures, or acinar-like arrangement of cells, changes which he believed might be presented by exfoliated mesothelial cells in fluids of long standing. Foot was correct in 60 per cent of the positive ascitic fluids and by using the nucleolar-nuclear ratio alone he was correct in 72 per cent. By utilizing this method, his record of accuracy for positive fluids fell far below those of Zemansky's but his results with negative fluids were appreciably more accurate. Quensel[3, 4] and Karp[5] emphasized that the ratio of nuclear to nucleolar size is high in cells of malignant tumors, thus agreeing with Foot. McCarthy* has emphasized, too, the difference in the nuclear-nucleolar area ratio of malignant and hyperplastic cells observed in unfixed tissue. As Helwig states, this is just the reverse of the nucleolar-nuclear ratio but gives the same ultimate results. Haumeder and McCarthy[1, 2] found the ratio of nucleus to nucleolus for the cells of regenerative breast to be 17 to 1 and for the malignant cells 11 to 1, whereas, Naidu[3, 4] working with similar organs in the same laboratory found the nuclear-nucleolar ratio for the cells of regenerative breast to be 21 to 1 and for malignant cells 17 to 1. Helwig states very properly that from these studies, it seems that the nucleolar-nuclear ratio is of least value in that group of fluids which is also most difficult to diagnose by a morphologic study of the cell.

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[1] Zemansky, A. P.: Am. J. M. Sc. 175: 489, 1928.

[2] Foot, N. C.: Am. J. Path. 13: 1, 1937.

[3] Quensel, U.: Acta med. scandinav. 68: 427, 1928.

[4] Quensel, U.: Acta med. scandinav. 68: 458, 1928.

[5] Karp, H.: Ztschr. f. Krebsforsch. 36: 597, 1932.

Helwig's general conclusions were that the report of "tumor cells present" should carry with it a high degree of accuracy and that a report of "no tumor cells present" is of less diagnostic value. Further, he believes that atypical mitotic figures and bizarre multinuclear cells are indicative of malignant cells. He warns against confusing desquamated cells from the lining of the serous cavities with tumor cells. Cells arranged in clumps or acini should be accepted as tumor cells only when they exhibit pronounced pleomorphism.

Summarizing: (a) Effusions resulting from carcinoma show "cancer cells" in a large majority of cases, together with considerable blood; (b) cardiac transudates show a predominance of mesothelial cells. Red blood cells are abundant; (c) tuberculous effusions shows a predominance of lymphocytes, with a few red cells.

In addition to carcinoma, one may find in the fluid other important cellular material as follows:

(a) In actinomycotic fluids one often finds the characteristic particles which consist microscopically of a mass of fine radiating fibers. These accumulations are often calcified and must be decalcified with diluted hydrochloric acid. They must not be confused with fatty acid crystals.

(b) Puncture fluids frequently show echinococcus cysts.

(c) In addition to the cellular elements already seen, crystals may be found, especially in old fluids. Chlosterine crystals are found in purulent breakdown of ribs; hematoidin crystals are also likely to be found.

ASPIRATION BIOPSIES

In an evaluation of biopsy by needle puncture, the following questions must be answered[5]: Do the results justify the means? What can be learned by this procedure that cannot be determined by other methods? If there is a choice, is aspiration biopsy the method of choice? Does the simplicity of the technical procedure outweigh the possible danger to the patient?

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*McCarthy, W. C.: Am. J. Cancer 26: 529, 1936.

[1] McCarthy, W. C.: Am. J. Cancer 26: 529, 1936.

[2] McCarthy, W. C., and Haumeder, E.: Am. J. Cancer 20: 403, 1934.

[3] McCarthy, W. C.: Am. J. Cancer 26: 529, 1936.

[4] Naidu, V. R.: Proc. Staff Meet., Mayo Clin. 10: 356, 1935.

[5] Smetana, Hans F.: Am. J. Clin. Path. 24: 395, 1954.

Needle biopsy as a diagnostic procedure has proved to be practical, valuable, and reliable. It should be used if there is a deep-seated lesion located beneath normal tissue where the damage resulting from the surgical approach of the operation would outweigh the advantages gained. It should be used if the procedure can save time or expense, or prevent inconvenience or discomfort to the patient, and can furnish information of practical value to the physician. It is to be recommended whenever there is danger of postoperative infection of the aspiration wound, possible fungation of tumor through the incision, particularly in lesions of bones, nasal sinuses, breast, and subcutaneous nonencapsulated tumors.

The most important contraindications are[2] abnormalities in the clotting or bleeding time; danger of spreading and disseminating a disease process or infection of body cavities; unjustified aspiration, promising only information of doubtful value or having little more than academic interest; and an uncooperative patient.

The technics for aspiration biopsy may vary somewhat for the organs or regions concerned. Ordinarily an 18 gauge, long needle is used, but special devices have been advocated and have been successfully used. They will be hereafter described. Usually after Novocain infiltration of the skin and soft tissue, the skin is nicked with a scalpel and the needle is inserted. A 20 c.c. syringe is attached to the needle after the desired organ is entered, a vacuum is produced in the syringe, the needle is slowly advanced, and then gradually withdrawn, the vacuum being slowly released before the needle is entirely removed in order to avoid splashing of tissue into the barrel of the syringe. After the needle is withdrawn, the tissue block is forced out of the needle by pressure on the plunger of the syringe. Spreads can be made from small fragments, while most of the tissue cylinder is fixed for histologic examination.

Necessarily, only small pieces of tissue can be obtained.

The criteria for diagnosis should be sharply and exactly defined. The examiner must know how to differentiate primary from secondary tumors. It is sometimes difficult to differeniate inflammatory or degenerative lesions from neoplasms.

Biopsy of Liver[3]

Liver biopsy has a definite place in the diagnosis of obscure hepatic diseases. This is well pointed out by Sherlock.[4] Her technic is as follows:

Preparation of the Patient.—Jaundiced patients are given vitamin K preparations for three days before the puncture. If the jaundice is nonobstructive (urobilin present in the urine), two 5 mg. tablets of "Kapilon" are given by mouth three times a day. If the jaundice is obstructive, the vitamin must be administered intramuscularly; 5 mg. daily is a suitable dose.

In every case, before biopsy is performed, the patient's blood group should be known, and two pints of compatible blood should be readily available. Aspiration liver biopsy should not be performed unless adequate facilities for blood transfusion are available in case of complicating hemorrhage. A sedative is unnecessary before puncture.

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[2] Martin, H. E., and Stewart, F. W.: Am. J. Roentgenol. 35: 245, 1936.

[3] Univ. Hosp. Bull., Ann Arbor 12: 13, Feb., 1946; Davis, W. D., Scott, R. W., and Lünd, H. Z.: Am. J. M. Sc. 212: 449-461, 1946.

[4] Sherlock, S.: Lancet 2: 397-401, Sept. 29, 1945.

Hepatic Puncture.—Have the patient lie supine in bed with the right side as near the edge of the bed as possible. A firm pillow may be placed under the left side in the hollow of the bed, so that the body is slightly tilted to the right. The right arm is placed behind the head. A site is chosen in the ninth or tenth intercostal space in the middle or anterior axillary line. After cleansing, anesthetize the skin with 2% procaine solution. Use a long fine-bore needle to infiltrate the pleura and then pass it through the diaphragm to anesthetize the peritoneum and the capsule of the liver. At least 10 c.c. of local anesthetic are needed. If the skin is tough, a preliminary nick may then be made with a scalpel. The cannula used is 15 cm. long and 1 mm. in bore. It is fitted with a handled trocar. Pass the instrument through the skin, and then instruct the patient to ''take a deep breath in, let it out, and then hold your breath.'' This displaces the lung upwards and insures apposition of diaphragmatic and costal pleura. Now push the trocar and cannula through the diaphragm into the right lobe of the liver. Do not withdraw the trocar until the instrument is fully half an inch inside the liver substance. Then punch out the cylinder of liver tissue by pushing the cannula on a further 4 to 5 cm. Connect a 20 c.c. ''Record'' syringe to the cannula and apply and maintain suction while withdrawing the cannula. Seal the puncture wound in the skin with collodion. The fragment of liver is usually found in the barrel of the syringe; occasionally it remains in the cannula. The aspiration of blood with the biopsy specimen need not occasion alarm.

Aftercare.—As a little local pain may follow puncture, morphine gr. ¼ or gr. ⅙ is given subcutaneously, according to the size and type of patient. This allays discomfort and prevents restlessness. If necessary, a further sedative, such as barbital soluble gr. 10, may be given in the evening. The pulse is charted hourly for the first 24 hours after biopsy; the physician should be called if the pulse rate shows a rise. Routine visits should be paid 4 and 8 hours after biopsy. A very careful watch must be kept on the patient, and if there is any sign of hemorrhage the cross-matched blood should be administered. Absolute rest in bed is essential for 24 hours. The patient can then be up and about and if desired can leave the hospital 48 hours after the liver puncture.

The procedure is attended with very little disturbance to the patient. During the puncture there may be a complaint of a drawn feeling across the epigastrium. Afterward some patients have a slight ache in the right side for about 24 hours, and some complain of a pain referred from the diaphragm to the right shoulder. Most patients agree that the discomfort compares favorably with that associated with sternal or lumbar puncture. Thirty patients had more than one biopsy; one had four.

Difficulties.—There may be failure to get an adequate sample of liver. Hoffbauer (quoted by Watson, 1944) had 40% failures with the Tripoli and Fader (1941) technic. Iverson and Roholm (1939) reported a 10 to 15% failure-rate; van Beck and Haex (1943), however, using the same method, state that the puncture failed only rarely. In Sherlock's first 126 biopsies there were 10% of failures; in the next 138 only 2%. Difficulties arise most often in hepatic cirrhosis, especially if there is associated ascites. In cirrhosis the tough liver is difficult to pierce and a few liver cells may be extracted leaving the fibrous framework behind. In ascites the liver is very ''ballottable'' and is difficult to transfix. A paracentesis abdominis should be undertaken before the liver biopsy is attempted, and the patient should lie well over on the right side during the puncture. This brings the liver into contact with the chest wall. Another source of difficulty may be pulmonary emphysema; the liver is pushed downward by the low diaphragm. It is very easy for the trocar to pass above the liver. If a low diaphragm is suspected before biopsy, the chest should be radiographed, and if necessary the puncture can be made through a lower intercostal space.

Inspection and Fixation of Biopsy Specimen.—Some information may be gained from inspection of the biopsy specimen. Fatty livers have a characteristic greasy look. Biopsy specimens of livers with excess fibrous tissue tend to crumble into fragments with a lobulated contour. If a malignant deposit has been punctured, the dull white appearance of the biopsy specimen is characteristic.

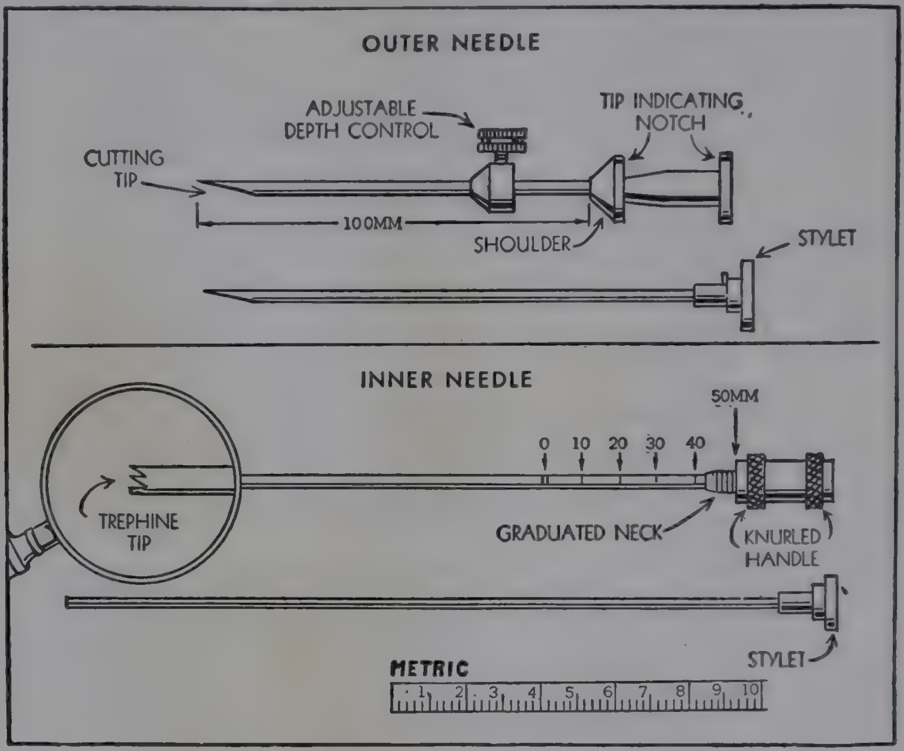

Fig. 297.—The Turkel standard liver biopsy set. (Courtesy Dr. Henry Turkel, Detroit, Mich.)

Fixation is best accomplished in absolute alcohol, which, while it dissolves the red cells, preserves the glycogen in the liver cells and enables Best's carmine stain to be applied. To demonstrate the elements of blood in the hepatic sinusoids, formol-saline is the most satisfactory fixative. Fat can be shown by fixing the material in Bouin's solution followed by 2% osmic acid. After fixation the specimen is embedded, sectioned longitudinally along the line of the cylinder, and stained in the usual way. If desired, the sample can be examined bacteriologically.

Reliability of Biopsy Specimens in Assessing Liver Histology.—Excluding the obviously localized conditions, such as malignant metastases, abscesses, and cysts, most other examples of human liver diseases have a reasonably uniform histology, and, therefore, biopsy specimens are likely to give good results. In Sherlock's experience, thirty cases came eventually to necropsy and in each the histology of the biopsy specimen was a fair sample of the liver as a whole.

Use of Biopsy Specimens in Diagnosis.—In regard to acute hepatitis, if the biopsy is performed early in the jaundiced phase, the acute liver inflammation presents a characteristic picture and diagnosis can be made. If the biopsy is postponed until convalescence, normal liver tissue may be observed and diagnosis is then impossible. In cirrhosis of the liver, clinical diagnosis may be aided by this liver biopsy study.

In obstructive jaundice, aspiration liver biopsy readily distinguished obstructive icterus of short duration from other types of jaundice.

In malignant disease of the liver without jaundice, very often a portion of a localized lesion is obtained for section, and it may indicate the site of the primary disease.

In connection with this information, attention should be called to the Turkel standard liver biopsy set. We recommend this for carrying out this technic. Fig. 297 shows the various parts of this equipment.

The outer splitting needle and stylet are forced through the skin, subcutaneous tissue, and liver; the liver is entered for a distance of about 5 mm. The stylet is replaced by the inner trephine needle which cuts the liver tissue to the desired depth. The inner needle is then removed and the biopsy tissue expressed with the stylet.

In connection with the use of this instrumentarium, the following **points on biopsy technic** must be observed:

1. Maintain asepsis
2. Instruments and materials:
    a. Liver biopsy set
    b. A 20 c.c. syringe (for retaining soft liver tissue within the inner needle)
    c. Abscess scalpel, syringe with local anesthetic (procaine)
    d. Biopsy sheet, dressings, antiseptics, gloves

Turkel suggests that *the most common points of insertion* are:

A. Anteriorly between mid-clavicular line and midline, just below the costal margin—only if the liver extends at least 4 cm. below the costal margin
B. Right mid-clavicular line—if liver is palpable 5 cm. below right costal margin
C. Between 9th and 10th rib on right side in anterior or mid-axillary line

In case of nodular liver where nodules are definitely palpable it might be advisable to take two specimens, one over the nodule and one in any non-nodular area for comparison.

Sherlock,* in her analysis of 264 biopsies, selected representative cases and gave six typical histologic pictures showing various diseases in which this method was used:

1. A typical case of hepatic cirrhosis following arsenotherapy jaundice.
2. Secondary hepatic squamous epithelioma with cell nests.

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*Loc. cit.

3. Histiocytic medullary reticulosis showing increased nucleated cells in the sinusoids and portal tracts.

4. Amyloid disease showing heavy infiltration of hepatic sinusoids with pale-staining amyloid.

5. A case of kala-azar showing histiocytic accumulations scattered through the liver.

6. Another case of kala-azar showing Leishman-Donovan bodies lying within the histiocytes.

Aspiration Biopsy of Lung and Material in the Pleural Cavity

According to Smetana, Menetrier is credited with having made the first diagnosis of cancer of the lung by histologic examination after needle puncture, in 1886.[1] Other available literature on the subject of needle biopsy of the lung will be found in the work of Dutra and Geraci.[2] They call attention to the extreme difficulty in ascertaining the nature of lesions in the peripheral portion of the pulmonary parenchyma. Roentgenographic evidence of the presence of a region of increased density within the lung is always an indication for additional diagnostic procedures. For this purpose, the frequently utilized examinations are smears of sputum for organisms and malignant cells, cultures for bacteria and fungi, and bronchoscopy. All of these may fail to provide a definitive diagnosis, especially when the lesion is located distal to the secondary bronchi. There has been lately a tendency toward thoracotomy with biopsy resection of the lesion in such circumstances. At times neither is desirable nor practical. Craver[3] described a technic by which a needle passed through an intercostal space was used to obtain a fragment of diseased tissue from peripheral pulmonary lesions. Dutra and Geraci believe that there are cases in which needle biopsy is a valuable procedure and, if used only when indicated, the danger of complications will be negligible in relation to the information obtained.

Craver's technic was the introduction of a short-bevel No. 18 needle into the mass, followed by attempts to aspirate groups of cells from the lesion with a syringe. Recently there has been described the use of the Vim-Silverman biopsy needle for this procedure. This procedure is a simple one.

Use of the Vim-Silverman Biopsy Needle.—Have on hand a Vim-Silverman biopsy needle.

Use a barbiturate and morphine or meperidine hydrochloride solution as a premedication.

Under fluoroscopic control, locate the tumor, and by rotation of the patient determine the point where the lesion is nearest the chest wall.

Mark this area of the chest wall with Merthiolate, light the room, and infiltrate the skin and subcutaneous tissues with 1% solution of procaine hydrochloride.

Insert the biopsy trocar and sheath through the skin and appropriate intercostal space in the direction of the tumor.

With the patient again before the fluoroscope, pass the trocar into the lung tissue and the periphery of the tumor mass.

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[1] Craver, L. F., and Binkley, J. S.: J. Thoracic Surg. 8: 436, 1939.

[2] Dutra, Frank R., and Geraci, Charles L.: J. A. M. A. 155: 21, 1954.

[3] Craver, L. F.: Surgery 8: 947, 1940.

Then withdraw the trocar from the sheath and insert the cutting blades down the sheath into the tumor mass.

Drive the blades forward to the hilt with a slight twisting motion. This carries them approximately 2 cm. beyond the sheath and into the tumor tissue.

Rotate the blades completely several times to sever the core of tumor tissue and then withdraw the blade. The core of tumor tissue is found between the blades.

Drop it into a 4% solution of formaldehyde (10% Formalin).

Needle biopsy of the lung should be employed when there is evidence to suggest the patient has a malignant tumor of the lung that could not be cured by resection. Its use should be restricted to cases in which other diagnostic methods have failed to identify the pulmonary lesion. The procedure should be done only in cases in which the following criteria are met: (1) the chest films reveal one or more opaque lesions in the peripheral pulmonary tissue; (2) examination of several smears and cultures of sputum and gastric washings have been done without isolating *Mycobacterium tuberculosis;* (3) evidence of a malignant neoplasm cannot be obtained by the technics of exfoliative cytology or by bronchoscopic examination and biopsy; (4) there is clinical evidence of incurability of the lesion.

Biopsy of Prostate*

Prostatic biopsy has been a much neglected procedure since it has not been possible to obtain a satisfactory specimen in many instances. The Turkel standard prostate biopsy set No. SBP 1214-100-50 consists of: One outer sheath or splitting needle gauge 12, 100 mm. long with its obturator or stylet; one adjustable depth control guard; one inner sheath or trephine needle gauge 14, extending 50 mm. with its obturator or stylet.

It serves as an excellent method of obtaining material from prostate for diagnosis.

Technic.—

Force the outer sheath or splitting needle and obturator through the skin until the tip of the needle partly enters the gland. Replace the obturator with the inner sheath or trephine needle which cuts the glandular tissue to the desired depth. Then remove the inner sheath and express the tissue.

If only biopsy is to be performed, the patient may be anesthetized with Pentothal sodium or by deep infiltration with a local anesthetic since the entire procedure may last only about five minutes.

If major operative procedures are contemplated, the patient may be given spinal anesthesia or whatever type of anesthesia may be decided upon.

There are certain points to be remembered; first, always maintain asepsis and, second, have all instruments and material ready for biopsy.

Assemble the following material:

Trephine prostatic biopsy set.
An empty 20 c.c. syringe for retaining fluid or soft tissue within the inner sheath.
Abscess scalpel, syringe with anesthetic.
Biopsy sheath, dressings, antiseptics.

The patient is put in the lithotomy position and sterile preparation of the entire field is done.

Make a nick with an abscess scalpel to facilitate entrance of the outer needle.

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*J. Urol. 51: 66, 1944.
Year Book of Urology, page 280, 1944.

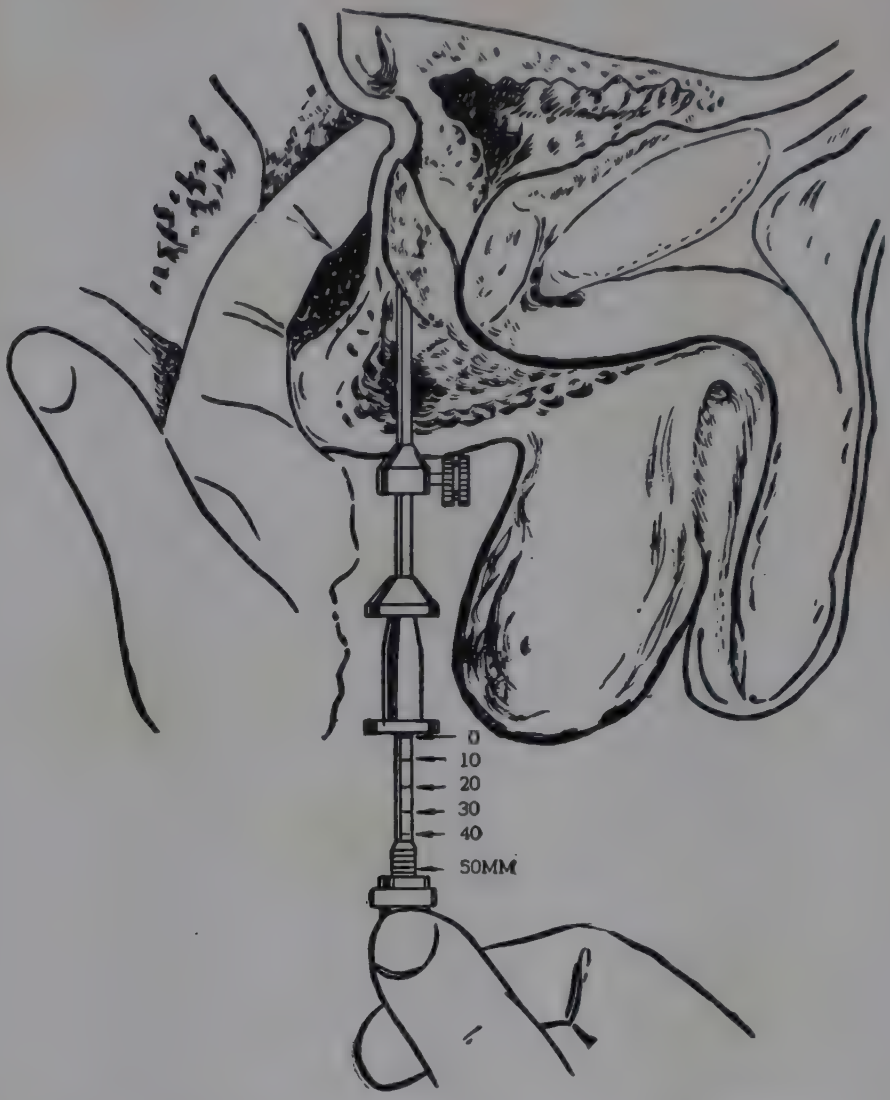

Fig. 298.—Aspiration biopsy of the prostate. The rectal finger is passed to the proximal portion of the prostate and hooked above. (Courtesy Dr. Henry Turkel, Detroit, Mich.)

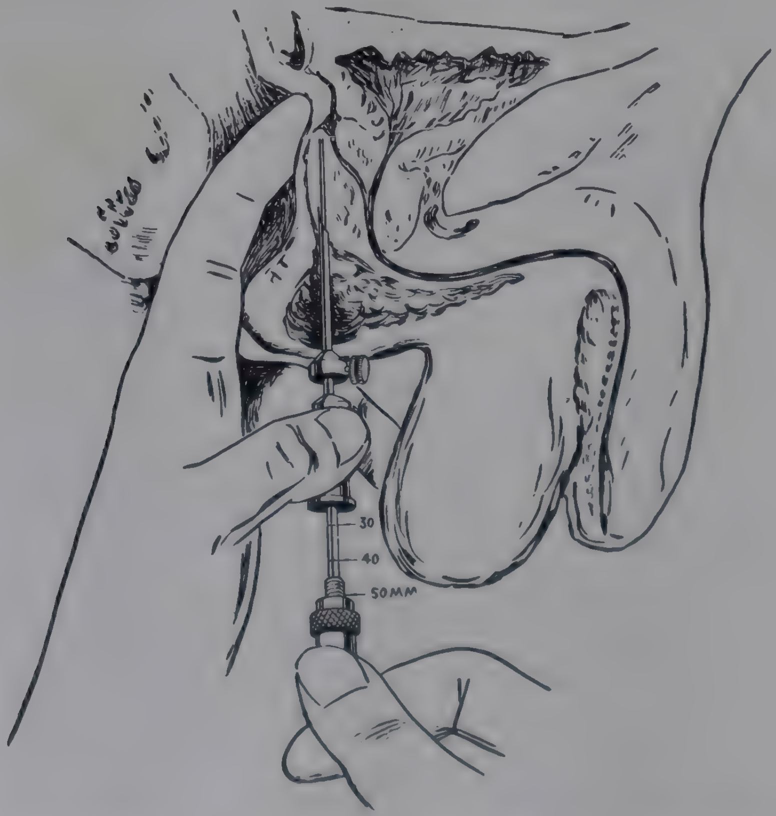

Fig. 299.—Aspiration biopsy of the prostate. Advance the inner trephine sheath through the gland with a to-and-fro motion. (Courtesy Dr. Henry Turkel, Detroit, Mich.)

With the index finger of the left hand in the rectum, the outer sheath with its obturator is inserted through the perineum and guided with the rectal finger until it just enters the prostate.

One may or may not move the depth control guard close to the skin and tighten the screw.

At this point, withdraw the obturator and insert the empty inner trephine sheath. It is important that the rectal finger now be passed to the proximal portion of the prostate and hooked above (Fig. 298). This is done in order to give counter-resistance to the cutting section.

Advance the inner trephine sheath through the gland with a to-and-fro motion, Fig. 299. When the desired amount has been cut, turn the trephine sheath with a clockwise rotatory motion in order to separate the specimen from the remainder of the gland.

Should the tissue (gland) be soft or contain fluid, suction should be applied with a 20 c.c. syringe to the inner trephine sheath in order to retain the tissue within the inner sheath.

Remove the inner sheath while applying suction. Insert the inner obturator into the inner sheath and force the specimen into a bottle containing a fixative.

Look for tissue in the outer sheath since the section occasionally falls into this portion during removal.

Cover the point of insertion with an antiseptic dressing. Occasionally there may be a slight bleeding but this can be disregarded because gland compression will stop it after removal of the sheath. Coagulation of the channel may be done through the outer sheath with a special electrode attached to a Bovie or similar apparatus.

Biopsy of the Sternum

Biopsy of the sternum is discussed on pages 937 ff.

Needle Biopsy of the Central Nervous System

Needle puncture of the central nervous system has been employed by many neurosurgeons as a routine procedure since the early part of the century. Unsatisfactory results of neurological examinations for determination of the nature and site of disease of the central nervous system are indications for its use. This applies to tumors, cysts, abscesses, hemorrhages, and other lesions, including disorders of the choroid plexus, tumors of the meninges, adenoma of the pituitary gland, glioma, neurinoma, and even capillary hemorrhages on an arteriosclerotic basis. It is also employed for therapeutic purposes, such as relief of increased pressure of the cerebrospinal fluid, localization and drainage of extradural hemorrhages, pachymeningitis, if the blood is still liquid, and drainage of abscesses and cysts. The danger of spreading the process through the central nervous system, for instance, during the puncture of a tubercle or an abscess, does exist; but such a complication is uncommon, according to Smetana.[1] The implantation of tumor tissue along the puncture canal is also possible. The greatest danger is infection of the meninges.

Aspiration Biopsy of the Spleen

With proper precautions, the application of acceptable technics, and correct assessment of the indications, splenic puncture can be regarded as devoid of danger if the procedure is restricted to enlarged organs (Smetana). It should never be carried out in patients suffering from hemorrhagic diathesis, in the presence of an acute splenic tumor, or in comatose patients. Splenic puncture should be performed only during full inspiration, and penetration into the pulp should not exceed 1 to 2 cm. The material obtained may be studied cytologically or histologically. It has many applications in the diagnosis of hematologic disorders, in metabolic diseases such as Gaucher's disease or Niemann-Pick disease, in kala-azar, in malignant lymphomas, in leukemias and other conditions. Together with bone marrow puncture, it can be of diagnostic value in some hematologic and metabolic conditions.

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[1] Am. J. Clin. Path. 24: 395, 1954.

Aspiration Biopsy of Bone

Aspiration biopsy of bone has certain advantages, namely, the simplicity of technic for obtaining material for study, the slight inconvenience to the patient who need not be hospitalized, and trauma of such minor degree that operations or treatment can be instituted at the site without waiting for wound healing.

If aspiration biopsy is contemplated for general conditions, such as myeloma, leukemia, and metastatic carcinoma, the iliac crest is regarded as the best site for aspiration. Sternal puncture (see pages 937 and 939) has been the method of choice for many workers, but we have had signal success by aspiration of iliac crest (see page 940).

Good diagnostic results of aspiration biopsy have been reported in cases of primary and secondary tumors of bone. Hodgkin's disease is not easily diagnosed by aspiration, nor is this method satisfactory in cases of osteitis deformans. The method may serve to exclude an apparent malignant tumor. Bone cysts are not suitable material. On the other hand, the Hand-Schüller-Christian, Gaucher, and Niemann-Pick diseases, eosinophilic granuloma, myeloma, and histiocytic lipomatosis can be diagnosed from material obtained by needle puncture.

Aspiration Biopsy of the Kidney

Aspiration biopsy of the kidney has been mainly used in the study of medical diseases, and therefore its use has been rather limited. It is thought to be a valuable adjunct, however, for the study of disorders rarely seen at autopsy in their active clinical phase. It is predicted that needle biopsy of the kidney will be instrumental in contributing to understanding such diseases as acute uremia, effects of shock, lower nephron nephrosis, and the effects of poisons. In this manner, the effects of damage to the kidneys can be evaluated and the histopathologic background correlated with the data on renal function. This also applies to the study of the natural history of the nephritides, hypertension of renal origin, interstitial nephritis, pyelonephritis, urolithiasis, and many other conditions of the kidney of which only the terminal phase is known and the pathologic picture of the early and intermediate stages is assumed.

The technic described by Iversen and Brun is the same as that for needle punctures elsewhere. X-ray examinations are essential to determine the presence and location of the kidney. An intravenous pyelogram with the shadow of the kidney outlined on the skin from at least two planes serves as a guide for the needle. The right side is preferred because of the presence of large venous channels on the left.

Iversen and Brun have reported success in 42 of 66 cases, with occasional transient hematuria 6 to 12 hours after puncture as the only complication.

Studies on renal biopsies were repeated by Parrish and Howe, using the Turkel biopsy needle, 12 gauge with a 75 mm. shaft and 100 mm. inner cutting needle. If there was abnormal clotting time or the prothrombin value was less than 70 per cent of normal, no biopsy was performed. The results reported by these authors were encouraging, being positive in 29 of 50 attempts in 41 patients.

These authors conclude that renal aspiration biopsy is a valuable adjunct in the diagnosis of renal diseases, and if necessary can be repeated in the same patient. It is contraindicated in congestive heart failures with elevated venous pressure, in bleeding diathesis, in the presence of only one kidney, and if perinephritis or perinephritic abscess is suspected. It is indicated in instances of undiagnosed renal disease, albuminuria, and hematuria of renal origin and obscure etiology.

Aspiration Biopsy of Miscellaneous Sites

Smetana describes the use of the Silverman biopsy needle in cases of suspected thyroiditis, struma lymphomatosa and Riedel's struma, in inoperable carcinoma, and in the study of the uptake of radioactive iodine.

Conventional biopsy of the pancreas is often unsatisfactory, because tumors are deep in the parenchyma and difficult to reach, the tissue is very vascular, inflammatory changes are frequent along the surface layers, and injury to ducts may produce a pancreatic fistula.

Occasionally aspiration biopsy has been performed on the testis and the epididymis for diagnosis of suspected tumor or other obscure clinical conditions. Similarly, such needle biopsies have been reported after puncture of masses found in soft tissue or in body cavities at operation, or of enlarged lymph nodes in various situations.

Summary

Aspiration biopsy constitutes a valuable means for securing histologic information concerning diseased conditions and the presence of neoplasms in many organs and areas. The indications vary somewhat for different organs and include inaccessibility of undiagnosed conditions for conventional exploration, after unsatisfactory results of clinical examination, search for the presence and type of neoplasms, follow-up studies of the course of disease, and the effect of treatment, and search for unrecognized systemic diseases. In addition, there are certain economic considerations.

The principal contraindications are the presence of states of hemorrhagic diatheses, the possibility of dissemination of infection, a spread and implantation of cancer cells, uncooperativeness of the patient, and lack of justification for, or poor chance of, success of aspiration.

CEREBROSPINAL FLUID

The examination of spinal fluid is a very important procedure in connection with many medical and surgical cases. For the method of withdrawing spinal fluid from the living subject, we are indebted to Heinrich Quincke who first treated two cases of hydrocephalus by means of withdrawal of the spinal fluid. In 1893 Lichtheim called attention to spinal puncture as a valuable aid in diagnosis, and in 1895, Fubringer reported 107 punctures in eighty cases. Since that time, spinal puncture has been generally adopted as a routine procedure for diagnostic and therapeutic purposes.

The ventricles of the brain which contain fluid slightly different in composition from that of the spinal fluid were first tapped by Middeldorpf in 1856. Occipito-atlantoid drainage of the cisterna magna in man was first performed by Parker in 1893. Cistern puncture has been developed mainly in this country. In 1919, Wegeforth, Ayer, and Essick performed the operation in man, using a needle for the puncture. Sicard, in 1923, was the first to introduce Lipiodol into the cistern as a roentgenographic aid in the diagnosis of cord tumors.

The cerebrospinal fluid is contained in the ventricles of the brain and in the subarachnoid space of the brain and cord. There are five ventricles, two lateral, and the third, fourth, and fifth. They are intercommunicating. The cerebrospinal fluid in the ventricles of the brain communicates with the fluid in the other ventricles through the various apertures connecting them. The only exception to this is the fifth ventricle which contains very little or no fluid. The fluid in the lateral ventricles flows into the third through the foramina of Monro; the fluid in the third communicates with that in the fourth by means of the aqueduct of Sylvius. The cerebrospinal fluid of the subarachnoid space communicates with the fluid in the ventricles of the brain through the foramen of Magendie which opens into the posterior part, and the two foramina of Luschka which open into the lateral recesses of the fourth ventricle and through numerous other apertures which open into the lateral ventricles. It is denied by some, however, that there is any communication between the cerebrospinal fluid in the ventricles of the brain and the fluid in the subarachnoid spaces of the brain and cord. But the best evidence that a communication exists between the ventricles of the brain and the subarachnoid spaces is the commonly observed fact that in hemorrhage into the ventricles the fluid obtained by lumbar puncture contains blood. It is also known that when a dye is injected into the ventricles, it can be recovered by lumbar puncture in a few minutes; and, vice versa, if the colored fluid is injected into the cisterna magna it can be recovered from the lateral ventricle.

In order to understand the diagnostic features of cerebrospinal fluid, something must be said of the physiology of this fluid. No one has yet determined the rate of formation under normal conditions. Equally uncertain are the data on the rate of absorption of the spinal fluid, but it has been shown that absorption probably occurs through the perivascular lymphatics directly into the blood stream.

The function of the cerebrospinal fluid is mechanical. The fluid forms a water bed around the cord and brain and prevents jarring from external trauma. It also equalizes the pressure between the brain and the cord. It is probable that the chorioid plexus, which is in the ventricles of the brain, is responsible for the secretion of the cerebrospinal fluid, although the evidence brought forward to prove this point is not altogether convincing. There is some evidence pointing to the assumption that the fluid is a direct product of the blood or of the brain tissue.

Method of Obtaining Spinal Fluid by Lumbar Puncture

Cerebrospinal fluid is obtained by lumbar puncture. The puncture is made between the third and fourth lumbar vertebrae. The type of needle used varies with different operators, but a strong needle 18 gauge, 3½ inches long suffices. The needle and stylet, which goes within the needle, are prepared as follows:

Place the needle with a small piece of cork inserted into the point into a test tube with a piece of gauze protruding over the edge of the test tube. Cork securely. Needles prepared this way are placed in the dry heat sterilizer and heated to 170° C. for two hours. They may then be kept indefinitely in these containers and are ready for use whenever required. Boiling is not recommended for the reason that it may not always kill spore-forming bacteria.

The patient is placed on his side on a firm operating table or in a bed. He is directed to draw up both knees and bow the head down so that the presenting vertebral column will have as much curve as possible, thus widening the spaces between the lumbar vertebrae so as to freely admit the spinal puncture needle. The so-called "soft spot" or opening between the vertebrae is found by the finger, and the entire area prepared with iodine followed by alcohol. The table and edges of the operating field are covered with a sterile sheet or towels. The operator with sterile rubber gloves now prepares the skin and subcutaneous tissue by local anesthesia, injecting a few drops of a 1 per cent solution of Novocain at the point selected for the puncture. In order that the spinal puncture may be successfully performed the patient's back must be at a perfect right angle to the plane of the bed or operating table, and the patient must be cautioned not to move from this position. With the finger, one finds the "soft spot" which is readily obtainable by dropping a line perpendicular from the crest of the ilium across the skin. The needle is then quickly inserted in the midline at a right angle to the long axis of the vertebral column and at a right angle to the lateral surface of the skin of the back. If the needle is properly introduced in the midline, it will go through the soft tissues quickly until it reaches the tough spinous ligaments where it meets with a little resistance. One can feel the "jump" of the needle as it leaves the tough tissues and punctures the dura of the spinal canal. The stylet of the needle is then removed, and if the puncture is properly made, fluid will begin to flow at once. If it does not flow, it is possible that the needle is blocked by a small piece of the dura. This can be dislodged by slowly and gently twirling the needle around.

Normal pressure of spinal fluid varies between 70 and 180 mm. of water. Values over 200 mm. are definitely high. Low pressures are found in patients with a subarachnoid block. High pressures are found in meningitis, brain tumor, brain abscess, and intracranial hemorrhage. Pressure is increased in patients with meningism, congestive heart failure, uremia, and aseptic meningeal reaction, also in neurosyphilis, cerebral trauma, and encephalomyelitis.

Under normal conditions, the spinal fluid comes out of the needle drop by drop; if the amount of pressure is very great, it may spurt out. The fluid may be collected in the sterile test tube in which the needle was prepared. It is well to have several other sterile test tubes on hand. The first few drops are received in the first tube and succeeding drops in the second and third tubes.

The presence of blood in the spinal fluid indicates either a traumatic injury above, or is the result of a faulty puncture. Faulty punctures are very common and are due to inexperience or to the use of needles that are too large. It is highly objectionable to contaminate the fluid with blood because this blood vitiates investigation for cytology, globulins, and even for the complement fixation tests. About 10 c.c. of fluid should be withdrawn for all purposes for which the fluid is tested.

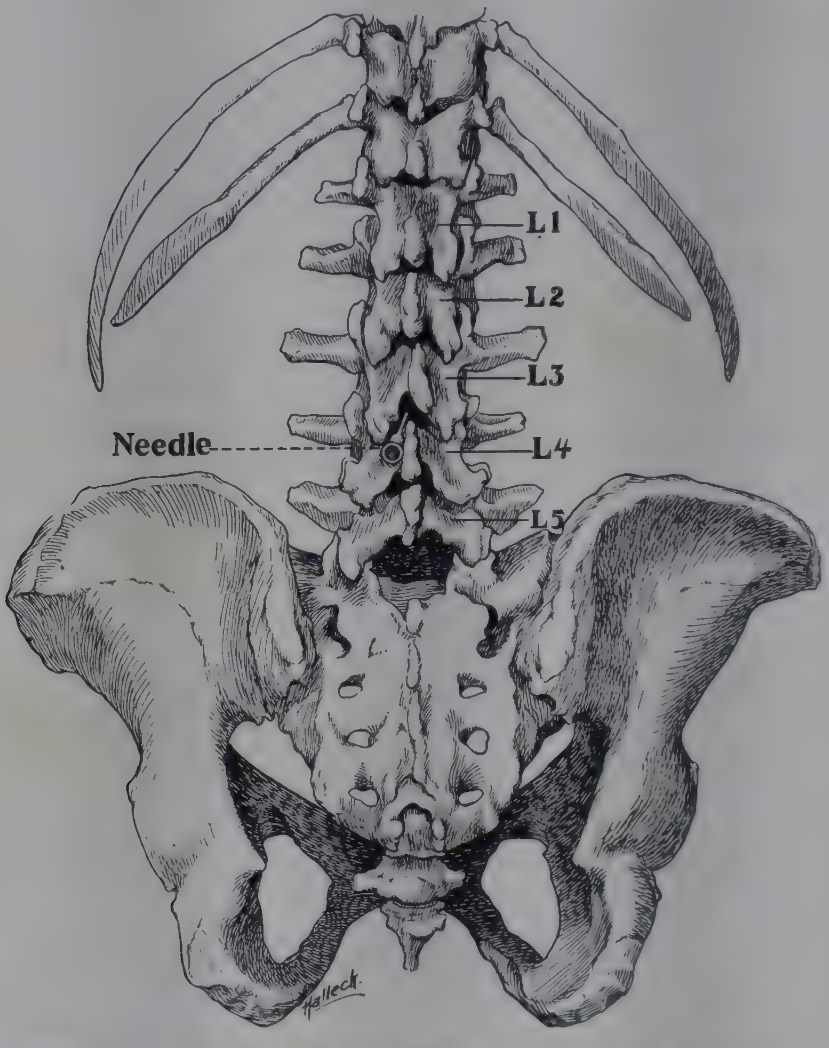

Fig. 300.—Position of the needle in a spinal puncture.

After the puncture, the patient is requested to lie in bed for twenty-four hours.

The reactions which follow spinal puncture vary in different individuals from a slight pain in the back of the neck to a very severe headache. This headache may be very persistent and last for a number of days, or it may disappear within twenty-four hours. There is no conclusive explanation as to the cause of this headache. Caution must be exercised in the practice of the spinal puncture on any patient suspected of having any new growth in the head, because the sudden release of spinal fluid below from a spinal puncture needle is likely to drive the contents of the cerebrum too suddenly against the foramen magnum with serious or even fatal results. In the presence of brain tumors especial care must be exercised in performing a spinal puncture.

The examination of the fluid should take place as soon as possible after withdrawal. It consists of the following tests: the *macroscopic* test; *bacteriologic* tests for tubercle bacilli, the various pus-producing bacteria (pneumococci, influenza bacilli, meningococci) and trypanosomes; *chemical* tests for globulin and sugar; *cytologic* examination for total white count, lymphocytes, and various cells which indicate disease changes; *serologic* examination, or complement fixation and precipitation tests for syphilis; the mastic test; and the Lange *colloidal gold* test. The study of the hydrogen ion concentration, cataphoreses, the Lange colloidal gold test, the ninhydrin reaction, and the precipitation tests, all of which are based on physiochemical principles, have clarified our understanding of the diseases of the central nervous system in general, and on the composition of the spinal fluid in particular.

If acute infections are suspected, complete bacteriologic examinations are necessary.

Characteristics of Cerebrospinal Fluid

Amount.—The amount of fluid normally present in the ventricles and in the subarachnoid space varies according to different authors from 60 to 150 cubic centimeters.

Color.—Normal cerebrospinal fluid is clear and colorless, provided no blood has been removed with it during the puncture. On standing it may become contaminated by bacteria or moulds, in which case it becomes turbid.

A very interesting condition which is called ''xanthochromia'' may sometimes be found. In this condition, the spinal fluid is a clear yellow color with rapid and marked coagulation, large amounts of globulin, and no blood pigments by chemical tests. It is generally the result of compression by a tumor of the cord or vertebrae with interruption to the flow of cerebrospinal fluid and formation of a pocket of greater or less extent in which the fluid stagnates.

Transparency.—Normal spinal fluid is clear. In acute meningitic infections, the fluid may vary from slight cloudiness to almost pure pus. It is absolutely clear in tuberculous meningitis.

Pellicle Formation.—In many acute cases of meningitis a pellicle or a net of fibrin and blood cells forms in the fluid on standing. In suppurative meningitis, the pellicle forms in a very short time; in tuberculous meningitis, it takes from twelve to twenty-four hours to form. Pellicles have been observed in poliomyelitis and in general paresis only rarely. The pellicle is formed by the separation of the heavier elements of the cerebrospinal fluid, so that microscopically one sees in the pellicle many leukocytes on a fibrinous background. Fig. 301 shows the forms of pellicle in various diseases as observed early, before autolysis takes place. There is considerable controversy as to whether or not the pellicle is dependent for its formation upon the presence of fibrinogen. The point has not been definitely established.

Lack of Sediment.—Normal fluid forms no pellicle or sediment. This lack of sediment is helpful in differentiating normal fluid from fluid in cases of meningitis in which a sediment is often present.

Specific Gravity.—The specific gravity varies according to different authors from 1.001 to 1.010.

Reaction.—Reaction of cerebrospinal fluid is alkaline, the alkalinity usually amounting to half that of the blood.

Levinson[1] states that the *hydrogen ion concentration* ranges from pH 7.4 to 7.6.

Total Solids.—Total solids vary between 1.200 and 0.876 gm. per 100 c.c.

Calcium.—Calcium content of the spinal fluid varies from 2.5 (figures of Depisch and Richter-Quittner) to 11.2 (Yvon) mg. of calcium in 100 c.c. cerebrospinal fluid.

Magnesium.—Magnesium content of spinal fluid is greater than that of blood, 3.0 mg. of magnesium to 100 c.c. of blood.

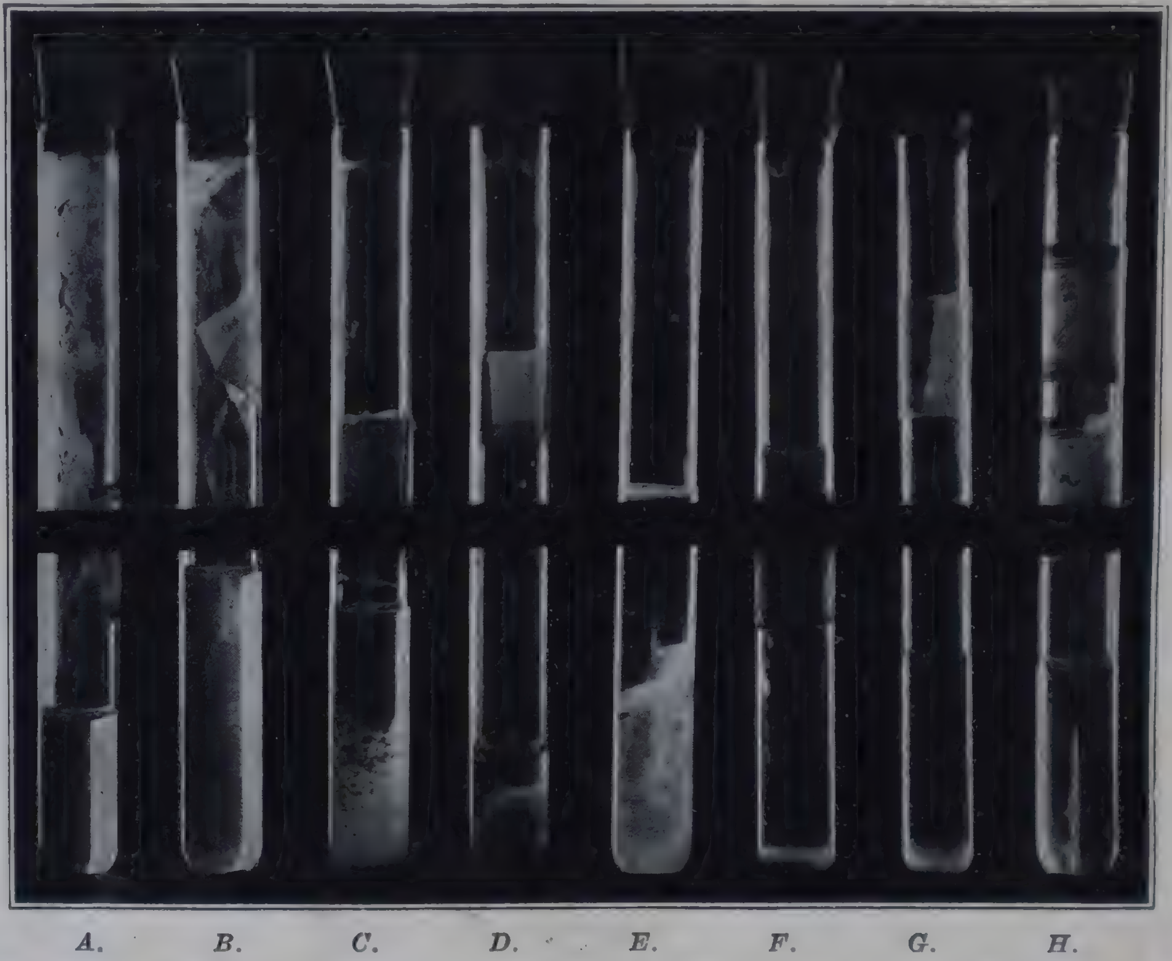

Fig. 301.—Pellicle-formation in meningitis. *A.* Normal. *B.* Meningococcus meningitis, fairly early. *C.* Meningococcus meningitis, advanced (sulphurlike granules). *D.* Meningococcus meningitis, late in disease. *E.* Meningococcus meningitis, severe, on road to recovery. *F.* Pneumococcus meningitis, fatal. *G.* Pneumococcus meningitis, fatal. *H.* Tuberculous meningitis. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.)

Chlorides.—Normal spinal fluid contains more chlorides, expressed as sodium chloride, than does blood plasma. The normal value for adults as NaCl is 720 to 750 mg. for each 100 c.c. of spinal fluid. In children, the normal range is from 625 mg. to 760 mg. per 100 c.c. The chloride content (in terms of Cl) is 390 to 460 mg. per 100 c.c., with a mean value of 450 mg.

The normal milliequivalent per liter is 110 to 130.

In acute meningitis, the amount of chloride may be reduced in adults to 625 mg. per 100 c.c. and to 575 mg. per 100 c.c. in children. In tuberculous meningitis, still smaller amounts are found. High values may be found in conditions in which the value for the plasma chloride is increased.

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[1] Levinson, Abraham: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.

Cytology of Spinal Fluid

Most writers are of the opinion that the normal cell count in spinal fluid varies from none to two per cubic millimeter. As a rule, we consider anything up to 5 normal.

Type of Cells Found.—The cells of spinal fluid are leukocytes, principally small lymphocytes. The nucleus of the lymphocytes of normal spinal fluid is round or slightly oval, seldom irregular. Large lymphocytes occur very rarely. Polymorphonuclear or segmented leukocytes are usually absent in normal fluids. The origin of the cells in normal spinal fluid has not been fully determined. The cell count should be made as soon after withdrawal as possible. Always shake the tube before diluting the spinal fluid.

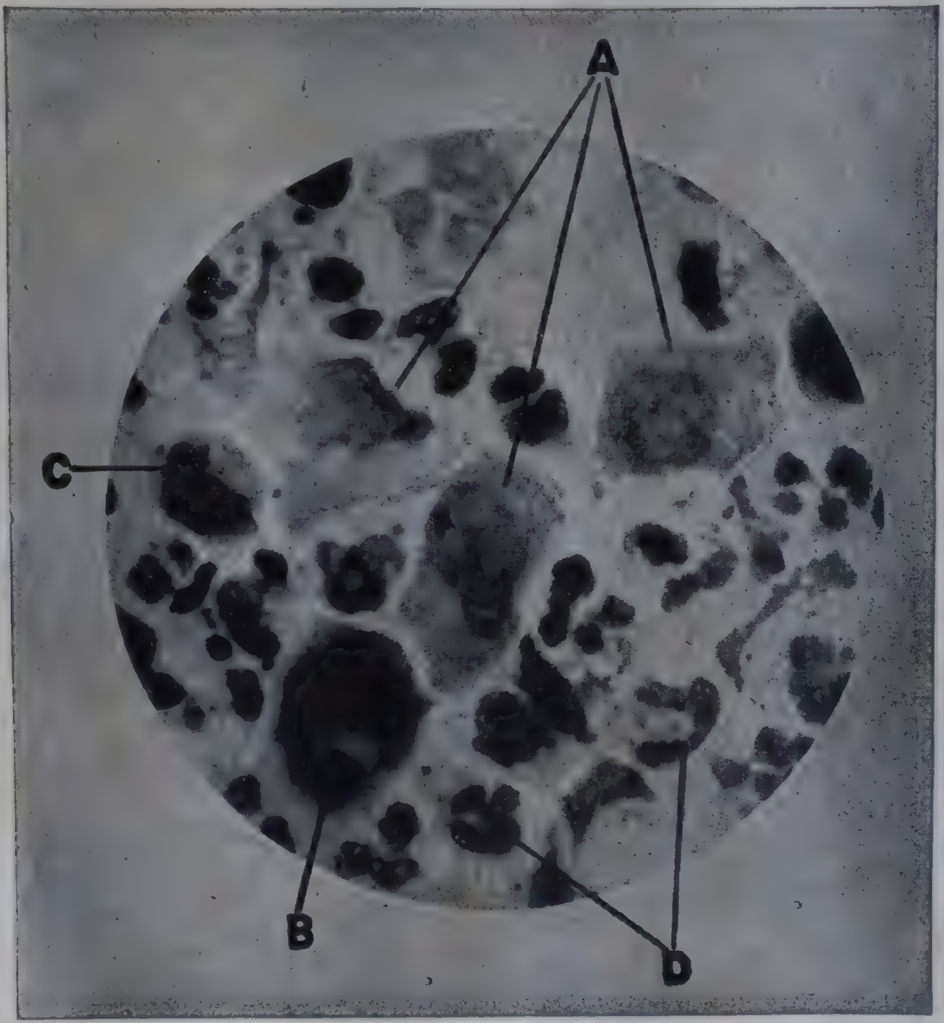

Fig. 302.—Photomicrograph showing types of cells in pathologic cerebrospinal fluid. (× 1,000.) *A*, Desquamated endothelial cells. *B*, Monocyte which has engulfed a polymorphonuclear leukocyte. *C*, Large mononuclear cell. *D*, Polymorphonuclear leukocytes. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.)

Method of Cell Counting in Spinal Fluid

Spinal Fluid Counting Solution (Tuerk's Solution).—

Glacial acetic acid	1 c.c.
Distilled water	99 c.c.

Add a few drops of alcoholic gentian violet to color. Filter several times.

Technic.—(With any counting chamber.)

Diluting the spinal fluid.—Draw the *spinal fluid counting solution* to the 0.5 mark and the *spinal fluid* to mark 11 in a white counting pipette. Mix thoroughly.

Filling the counting chamber.—Blow out two or three drops of the diluted spinal fluid before filling the counting chamber. Place the cover glass over the ruled area.

Allow the diluted spinal fluid to run under the cover glass so that it exactly fills the space between the cover glass and the counting chamber.

Making the count. Focus under the low power dry objective without the Abbé condenser.

Count all the cells in the entire ruled area which represents nine fields.

Divide by 9 and multiply by 10 to obtain the number of cells per cu. mm.

Make similar counts on two more drops of diluted spinal fluid.

Average the three counts for the report.

To correct for the slight dilution of the spinal fluid, divide the final result by 19 and multiply by 20. Do not report a fraction of a cell.

Example.—

1st drop	2d drop	3rd drop
9 | 18	9 | 20.0	9 | 18
2	2.2	2
10	10	10
20	22.	20
22		
20		
3 | 62		
19 | 20.6		

1.08 × 20 = 21.6 or 22 cells per cu. mm. of spinal fluid.

Normal 0 to 5 cells per cu. mm. (see below).

Counting of Spinal Fluid Contaminated by Blood.—The problem of whether or not an accurate cell count can be made of bloody spinal fluid may be answered in the following words—to date no reliable or absolutely accurate method can be recommended for estimation of pleocytosis in the cerebrospinal fluid if the fluid is contaminated by blood. An attempt has been made to do this by staining the fluid lightly with Unna's polychrome methylene blue and calculating all of the red cells and all of the white cells present in one cubic millimeter. Correction for the number of white cells present in the fluid as a result of the blood can then be made on the basis of the red and white blood count in the peripheral vessels. According to Merritt,[1] "while this method is not absolutely accurate there is usually no trouble determining a pleocytosis in the cerebrospinal fluid of twenty-five or more cells."

The author does not care to make a diagnosis of pleocytosis in bloody spinal fluid.

Making the Differential Count of Spinal Fluid.—This count can be made if there is a total blood cell count of over 500 cells per cu.mm. If the cell count is below this, the sediment may be procured by centrifuging the fluid in an open bottom centrifuge tube with a rubber stopper in the bottom. By centrifugalization the cells are thrown down onto the rubber stopper.

Technic of procuring the cells is to discard the supernatant fluid and spread the cells on a glass slide. In either case, whether the direct count or count of this centrifuged specimen is to be made, proceed in exactly the same manner in which a differential count of the leukocytes in blood is made.

As stated above, the normal cell count in the spinal fluid is 0 to 10 cells per cu.mm. in adults. In children under one year of age, 30 cells would be considered normal. From one to four years, 20 cells are found, and from five years to puberty, 10 cells.

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[1] Merritt, H. Houston: Personal communication.

Increased cell count is due to inflammatory lesion of the brain, spinal cord, or meninges. An increased count is found in cerebral tumors but no relationship can be drawn between the type or location of the tumor and the cell count. An acute process will show an increase in the segmented neutrophiles. This is especially true of pus infections. In chronic infections and toxic conditions, there is usually an increase in lymphocytes. A slight increase, say from 10 to 100, especially of the lymphocytes, occurs in early tuberculous meningitis, neurosyphilis, anterior poliomyelitis, encephalitis lethargica, and multiple sclerosis. As many as 1000 cells, principally lymphocytes, have been found in late tuberculous meningitis, poliomyelitis, and acute syphilitic meningitis. The author recalls a case of paresis many years ago with a cell count of 600. Naturally, over 1000 cells are found in purulent meningitis, influenza meningitis, and brain abscess. There is no increase in cells to any extent in uremic or diabetic coma.

Tests for Protein

These tests are commonly called tests for globulin, but they are really tests for protein since the methods used depend upon the precipitation of both albumin and globulin.

Normal concentration of cerebrospinal protein is between 15 and 40 mg. per 100 c.c. of fluid. In inflammatory conditions of the meninges, the choroid plexus, which is the barrier of the passage of the proteins, breaks down and permits these to enter the spinal fluid. In some other conditions, such as typhoid fever, uremia, and convulsive states, protein fluids are found. The increase in the proteins of the spinal fluid in suppurative diseases of the meninges is due to the accumulation of the protein of the polymorphonuclear leukocytes. In acute suppurative meningitis, protein concentration values may be as much as 1300 mg. per 100 c.c. Tuberculous meningitis shows values between 200 and 1000 mg. In tetanus there is an increase in the protein concentration, and an increase has been noted shortly after epileptic seizures. In such syphilitic diseases as paresis, tabes dorsalis, and other forms of cerebrospinal syphilis, values between 20 and 200 mg. have been reported. In epidemic encephalitis, protein concentrations vary between 50 and 300 mg. per 100 c.c. In cerebral hemorrhage, thrombosis, or embolism, there is an increased protein content. There are increased protein contents in brain abscesses and brain tumors, also in multiple sclerosis. In the disease known as "the Froin syndrome," in which there is complete obstruction of the meninges of the spinal cord, there is a difference noted in the protein concentration above and below the point of obstruction. For instance, a lumbar puncture may show in such a case as much as 1000 mg. per 100 c.c., and fluid taken from a cisternal puncture may show practically normal values. In myxedema, protein varies between 30 and 220 mg. Table 133[1] shows the changes in the pathologic conditions in respect to protein concentrations.

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[1] Quoted from "Synopsis of Applied Pathological Chemistry" by Andes and Eaton, page 248, The C. V. Mosby Co., St. Louis, Mo., 1941.

TABLE 133.—CEREBROSPINAL FLUID PROTEINS

CONDITION	CHANGES IN SPINAL FLUID PROTEIN TO BE EXPECTED	CONCENTRATIONS OBSERVED (IN MG. PER 100 C.C.)
Normal	0	20-40
Pathological:		
Brain tumor or abscess	+	30-100
*Cerebral hemorrhage	+	20-220
*Cerebral thrombosis	+	20-140
*Cerebrospinal syphilis	+	20-160
Epilepsy	+	20-80
*Epidemic encephalitis	+	50-300
*Froin syndrome	++	100-2100
Meningitis:		
*(a) meningococcic	++	150-750
*(b) pneumococcic	++	180-1300
*(c) streptococcic	++	150-900
*(d) tuberculous	+	50-200†
*Multiple sclerosis	+	
Myxedema	+	30-220
*Paresis	+	30-190
Serous meningitis (from influenza, otitis media, mastoiditis, pneumonia, typhoid fever, uremia, etc.)	0 to +	20-90
Spasmophilia or tetany	+	
*Tabes dorsalis	+	40-200
Tetanus	+	
Uremic convulsions	+	

*Diseases in which the determination may be of clinical value.
†Values as high as 1000 have been reported but such concentrations are rarely found.

The first test described below, the Nonne-Apelt or Ross-Jones test, is a test for globulin, while the Pandy and Noguchi tests are really protein tests.

A. QUALITATIVE TESTS

Nonne-Apelt Method (Ross-Jones)

Reagent.—

Nonne-Apelt (Ross-Jones) Reagent.—Saturated solution of ammonium sulphate, filtered after saturation has been reached.

Technic.—

Place 1 c.c. Nonne-Apelt (Ross-Jones) reagent in a test tube.
Stratify 1 c.c. spinal fluid on the reagent.
A white ring at the point of contact indicates the presence of globulin.
If the spinal fluid contains blood, the test cannot be made.

Pandy Method

Reagent.—

Pandy's Reagent.—

Dissolve 10 gm. of carbolic acid crystals
in 150 c.c. distilled water.
Place in the incubator and shake frequently for several days. Filter. The reagent must not become turbid upon shaking. It may become pink.

Technic.—

Place 1 c.c. Pandy's reagent in a test tube. Add a few drops of spinal fluid. Shake. A turbidity develops if globulin is present.

Noguchi's Method

This test is so delicate that it often detects globulin in normal spinal fluids.

Reagent.—

Butyric acid	10 parts.
0.9% sodium chloride	90 parts.

Technic.—

In a small test tube place 0.5 c.c. of Noguchi's reagent.

Add 0.1 c.c. spinal fluid.

Bring to a boil over a flame.

Add 0.1 c.c. N/1 NaOH.

A flocculent precipitate appears in a few minutes if globulin is present in large quantities. The precipitate may take a few hours to form, if the quantity of globulin is low.

Normal spinal fluids show a slight opacity. Traces of proteins are normally present in spinal fluid. A great increase occurs in acute inflammations, in tuberculous meningitis, and in syphilitic and parasyphilitic affections.

The chief protein is globulin, tests for which are outlined above. Because of the presence of serum globulin in blood, a globulin test is valueless if the spinal fluid is contaminated with blood.

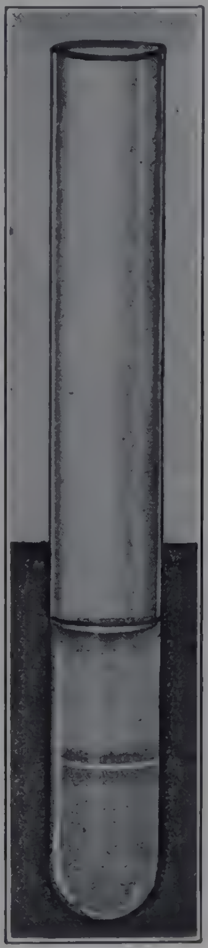

Fig. 303.—Nonne-Apelt test for globulin in spinal fluid, positive reaction.

The Weichbrodt Method

To 0.7 c.c. of spinal fluid in a test tube, add 0.3 c.c. of a 1% solution of bichloride of mercury.

A positive reaction is seen by opalescence or cloudiness.

Alcohol Test

A drop of cerebrospinal fluid is added to a test tube containing 0.5 c.c. of 95% alcohol. A very slight turbidity is normal, but a heavy white cloud or flocculation indicates a high protein content.

B. QUANTITATIVE TESTS

Method I

Technic.—

Measure 2 c.c. of spinal fluid into a 15 c.c. centrifuge tube, and add 3 c.c. water and 1 c.c. 20% trichloroacetic acid. Centrifuge out the protein precipitate, and pour off the supernatant fluid. Dissolve the precipitate in 0.25 c.c. of 10% sodium hydroxide and heat one-half hour in a water-bath. Then add 3.75 c.c. water, 0.5 c.c. Folin phenol reagent (page 305), and 1.5 c.c. saturated sodium carbonate solution. Mix by inversion, and read in a half hour in a colorimeter, setting the standard at 10 mm.

Standard.—Two standards should be made, one containing 1 c.c. 20 mg. % tyrosine and 7 c.c. water, and the other 2 c.c. standard and 6 c.c. water. Then add 1 c.c. phenol reagent and 3 c.c. saturated sodium carbonate solution to each tube.

Calculation.—

Standard 1

$$\frac{10}{B} \times \frac{0.2}{2.0} \times \frac{100}{2.0} \times 13.0 = \text{mg. protein per 100 c.c.}$$

$$\text{or} \quad \frac{650}{B} = \text{mg. protein.}$$

Standard 2

$$\frac{10}{B} \times \frac{0.4}{2.0} \times \frac{100}{2.0} \times 13.0 = \text{mg. protein per 100 c.c.}$$

$$\text{or} \quad \frac{1300}{B} = \text{mg. protein.}$$

Method II

Reagents.—

Standard Protein Solution.—Dilute 20 c.c. of normal human blood serum to 200 c.c. with a 15% solution of sodium chloride.

Filter.

Estimate total nitrogen of this filtrate by macro-Kjeldahl method, using 40 c.c.

Estimate nonprotein nitrogen in the original undiluted serum by the micro-Kjeldahl method of Folin. Divide this figure by 10 and subtract from the total nitrogen to obtain the protein nitrogen content of the concentrated standard solution. The protein nitrogen multiplied by 6.25 gives the protein content of the concentrated standard solution. Dilute concentrated standard solution with sufficient distilled water to make a dilute standard solution containing 30 mg. of protein per 100 c.c. Add thymol crystals and keep in refrigerator.

Sulphosalicylic Acid.—Dissolve 5 grams of sulphosalicylic acid, Merck, in 100 c.c. distilled water.

It has been recommended by Irvine[1] that this estimation can be carried out very conveniently by using small detachable cups and plungers in the Duboscq colorimeter or any other standard colorimeter. In other words, these attachments consist of a pair of all-glass cups of 1 c.c. capacity, solid glass plungers with a threaded adaptor, and holders for 1 c.c. cups. These parts are easily interchangeable with the standard ones. A special diaphragm with a smaller opening is used in the eyepiece.

Technic.—

This method has been described by Ayer, Dailey, and Fremont-Smith.[2] This is a modification of the method previously described by Denis and Ayer.[3]

Place 0.6 c.c. of cerebrospinal fluid in a test tube.

Add 0.4 c.c. of distilled water.

Add 1 c.c. of a 5% solution of sulphosalicylic acid.

Mix by inversion.

Let stand five minutes.

Read the turbidity in the colorimeter against that produced by a standard protein suspension prepared at the same time as the unknown.

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[1] Irvine, M. D.: The Cerebrospinal Fluid. Merritt and Fremont-Smith, p. 259. W. B. Saunders Company, 1937, Philadelphia.

[2] Ayer, J. B., Dailey, M. E., and Fremont-Smith, F.: Arch. Neurol. & Psychiat. 26: 1083, 1931.

[3] Denis, W., and Ayer, J. B.: Arch. Int. Med. 26: 436, 1920.

Preparation of the standard protein suspension:

Mix 3 c.c. of the dilute standard solution containing 30 mg. of protein per 100 c.c. and 3 c.c. of a 5% solution of sulphosalicylic acid.

Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times \frac{30 \text{ (number of mg. in 100 c.c. of standard)}}{\text{c.c. of spinal fluid used}} = \text{mg. protein per 100 c.c.}$$

Method III

Kingsbury, Clark, Williams, and Post described a method for the quantitative determination of albumin in urine. The method is very satisfactory for the quantitative determination of total protein in the spinal fluid.

Equipment.—

The equipment consists of a set of standard tubes filled with clear gelatin mixed with varying amounts of Formalin, which represents the turbidity produced by mixing a 3% solution of sulphosalicylic acid with known amounts of protein in serum. The standards keep for some years, although they should be checked from time to time for accuracy. They may be prepared according to the method which has been described by the originators of the method (see page 148).

Reagent.—

3% Sulphosalicylic Acid.—3 gm. in 100 c.c. of distilled water.

Technic.—

Place 1 c.c. of spinal fluid in a tube measuring ½ by 4 inches.

Add 3 c.c. of the reagent and mix thoroughly.

Allow the mixture to stand 5 minutes and compare the degree of turbidity with that of the permanent standard tubes.

The **normal amount of protein** in spinal fluid is usually about 30 mg. per 100 c.c.

Quantitative Test for Chlorides

Method of Osterberg and Schmidt

Reagents.—

Silver Nitrate Solution.—

(1 c.c. is equivalent to 1 mg. of chlorine.)

Dissolve 4.791 gm. of chemically pure silver nitrate in distilled water, and dilute to 1 liter in a volumetric flask.

Preserve in a brown bottle.

Potassium Thiocyanate Solution.—

Dissolve about 3 gm. of potassium thiocyanate in 1 liter of distilled water. Standardize against the silver nitrate solution so that 5 c.c. exactly equal 5 c.c. of the silver nitrate solution. Refer to page 434.

20% Ferric Ammonium Sulphate Solution.—

Dissolve 20 gm. ferric ammonium sulphate in distilled water and dilute to 100 c.c. with distilled water.

Dilute Nitric Acid Solution.—

Add 1 part of concentrated nitric acid, c.p., to 3 parts of distilled water.

Technic.—

Place 1 c.c. of spinal fluid in a 125 c.c. Erlenmeyer flask.

Add slowly 10 c.c. of dilute nitric acid from a burette, agitating the flask to form a white, flocculent preipitate.

Add 5 c.c. of silver nitrate solution and 1 c.c. of 20% ferric ammonium sulphate.

Titrate the excess of silver nitrate with the solution of potassium thiocyanate.

Calculation.—

5 − c.c. of potassium thiocyanate used × 100 = mg. of chlorine per 100 c.c.

To obtain the mg. of sodium chloride per 100 c.c. of spinal fluid, multiply by 1.65.

To change to meq./L., consult page 28.

Normal.—See page 1242.

Mastic Test (Cutting)

The mastic test has been proposed as a substitute for the gold test, but it is not as useful a test in the differentiation of the various types of syphilitic diseases of the nervous system. Complete precipitation of the mastic corresponds to complete decolorization of the colloidal gold reagent; while partial precipitation of the mastic corresponds roughly to the purple or blue of the colloidal gold test. The method used by Cutting follows:

Reagents.—

Mastic Solution.—

Gum mastic, U.S.P.	0.20 gm.
Absolute alcohol	20.0 c.c.
Freshly distilled water	80.0 c.c.

Dissolve the gum mastic in the absolute alcohol and centrifuge to clear.

Pour the filtrate rapidly into the distilled water and mix well.

Alkaline Saline Solution.—

Potassium carbonate, reagent grade	5.0 *mg.* (0.005 gm.)
Sodium chloride	1.25 gm.
Distilled water	100.0 c.c.

Dissolve the salts in the water.

Technic.—

Arrange a series of six small test tubes. In the first, place 1.5 c.c. of the alkaline-saline solution and in each of the others place 1 c.c. To the first tube add 0.5 c.c. of the spinal fluid, which must be completely free from blood. Mix by sucking the fluid up into the pipette and expelling it, and transfer 1 c.c. to the second tube. Again, mix and transfer 1 c.c. to the third tube and continue down to the fifth tube, discarding the 1 c.c. portion which is removed from this, and leaving the sixth tube with alkaline-saline solution alone to serve as a control. Finally add 1 c.c. of the mastic solution to each tube. Mix well and set aside at room temperature for twelve to twenty-four hours, or in the incubator for six to twelve hours. Tubes in which the reaction is complete will show a heavy precipitate with clear supernatant fluid.

Interpretation.—As stated before, this reaction is read as we read the Lange colloidal gold test reaction. If there is no precipitation of the colloidal suspension in any of the tubes, this is a negative reaction. Any precipitation is considered a positive reaction and the characteristics of the precipitation in the various tubes determine the interpretation of the localization of the syphilitic process; e.g., most precipitation in the first tubes is read as paretic zone; precipitation in the center tubes as luetic zone; and in the last tubes as meningitic zone.

Sugar Estimation in Spinal Fluid

It is generally believed that the sugar values in spinal fluid vary between 45 and 85 mg. per 100 c.c. Estimation of the amount of sugar in spinal fluid is important because a low sugar content is diagnostic of certain conditions.

For instance, there is a low sugar content in acute and subacute meningitis due to the glycolytic action of the organisms present. It also has been noted that there is a low sugar content in cerebrospinal fluid containing blood. This is due to the action of glycolytic ferments which are present in the blood, which serve to reduce the normal sugar content of the fluid. A low sugar content has been seen in cases of so-called aseptic meningeal reaction due to the glycolytic action of cells in vivo.

Technic.—

One may use the same method described elsewhere in this book by Folin and Wu for glucose determination in blood.

Reagents.—

Five Per Cent Sodium Tungstate Solution.

One-Third Normal Sulphuric Acid Solution.

Standard Sugar Solution, No. I.—See page 231.

Alkaline Copper Solution.—See page 230.

Phosphomolybdate Solution.—Place 35 gm. of ammonia-free molybdic acid, 5 gm. sodium tungstate, 200 c.c. of 10 per cent sodium hydroxide solution, 75 c.c. water in a Pyrex beaker. Boil vigorously until the fumes coming off fail to turn red litmus blue. Cool. Add 125 c.c. of concentrated (85 per cent) phosphoric acid. Dilute to 500 c.c. Be sure that any glassware used in making this solution, which has been previously contaminated with copper, is washed with nitric acid and rinsed thoroughly with water before being used.

Technic.—

Place 2 c.c. of the protein-free filtrate in a Rothberg-Evans sugar tube.

Place 2 c.c. standard solution in a similar tube.

To each tube add 2 c.c. of alkaline copper solution.

Boil for 8 minutes in usual way.

Place in cold water bath.

Add 2 c.c. of phosphomolybdate solution at once.

Cool.

Dilute the standard to 25 c.c. and the unknown to match it.

In cases where the sugar content is very low, one may make the standard dilution up to 50 c.c.

Read in a colorimeter. Set the standard at 20 and read the unknown.

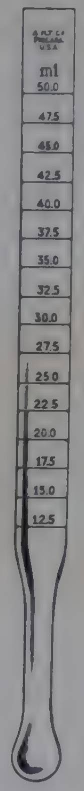

Fig. 304.—Rothberg-Evans tube.

Calculation.—

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{Dilution of unknown}}{\text{Dilution of standard}} \times 10 \times 10 = \text{mg. glucose per 100}$$

c.c. cerebrospinal fluid.

Be sure to make the sugar determination as soon as possible after the spinal fluid has been removed.

Lange Colloidal Gold Test

The method which is recommended for the preparation of Lange's colloidal gold solution is that suggested by Levine.[1] He had previously[2] described a method for this preparation using gold chloride, potassium carbonate, and formaldehyde. At that time, he suggested the use of triply distilled water and a block-tin or resistant-glass distilling assembly. In 1934, Borowskaja[3] found that sodium citrate could be used in the preparation of gold sol instead of the potassium carbonate and the formaldehyde. She found, further, that sodium citrate stabilized the reaction sufficiently so that once-distilled water could be employed with safety. Levine made a study of Borowskaja's reaction and found that the time which it took for the reaction to reach its completion varied in duration from five to thirty minutes and occasionally even longer, and that the sensitivity of the final sol to precipitation by positive spinal fluids varied accordingly. But he found that by the addition of a few drops of a fresh solution of 3 per cent hydrogen peroxide instead of bubbling air or oxygen through the hot water, the reaction began immediately and reached completion within a few seconds. The method which is here advocated is that originated by Levine.

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[1] Levine, B. S.: Venereal Disease Information, February, 1939.

[2] Levine, B. S.: Am. J. Syph. 16: 103, 1932.

[3] Borowskaja, D. P.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 82: 178, 1934.

Glassware should be chemically cleaned. While it is recommended that once-distilled water be adjusted to pH 7.2 to 7.4, this is not essential.

Technic.—

To prepare 500 c.c. of gold sol the following formula is recommended:

Distilled water	460 c.c.
Gold chloride (1% solution)	5 c.c.
Sodium citrate (1% solution)	25 c.c.
Hydrogen peroxide (fresh 3% solution), 3 to 5 drops, diluted in 10 c.c. of distilled water.	

Heat the distilled water to brisk boiling.
Remove from the hot plate.
Add the 5 c.c. of the 1 per cent gold chloride solution.
Shake for a few seconds.
Add the 25 c.c. of the 1 per cent sodium citrate solution.
Shake for 10 or 15 seconds.

While shaking, add the 10 c.c. of distilled water which contains the five drops of hydrogen peroxide. Continue shaking for a few seconds. Let the flask rest on the table until the reaction is completed. This usually takes from one-half to one minute.

The advantages of this method are resident in the fact that, first, it is not necessary to use triply distilled water; second, that the reaction of the water does not have to be adjusted; third, this method can be used to make as little as 100 c.c., can be made with the same ease and with as high a degree of reliability as larger volumes. In Levine's experience, however, the reaction proceeds in a more regular manner in large volume manufacture, say, 500 or 1000 c.c.

Note: The prepared solution ordinarily requires no special treatment for neutrality. It is advisable, however, to test it with known normal and known paretic spinal fluids. It must give no reactions beyond the red-blue color with a known normal spinal fluid, and a typical paretic zone curve with the known paretic spinal fluid. The neutrality of the colloidal gold solution must be controlled; this may be carried out with alizarine red. Place 10 c.c. of the colloidal gold fluid in a clean flask, add one or two drops of a 1% solution of alizarine red in 50% alcohol. Titrate with N/50 sodium hydroxide until neutral. With this solution of alizarine, acid reaction shows lemon yellow; neutral, brown red; alkaline, reddish purple. After estimating the quantity of acid or alkali necessary to neutralize the 10 c.c. sample, add enough of one or the other to neutralize the entire amount of colloidal gold reagent that has been prepared.

A special set of glassware should be kept for the making of the reagent and the test. This glassware should be chemically cleaned, and care should be exercised in preventing contamination of the spinal fluid with dyes or chemicals before it is tested. Glassware should also be cleaned in 50% **aqua regia** (3 parts HCl, 1 part HNO_3, 4 parts water) to remove precipitated gold.

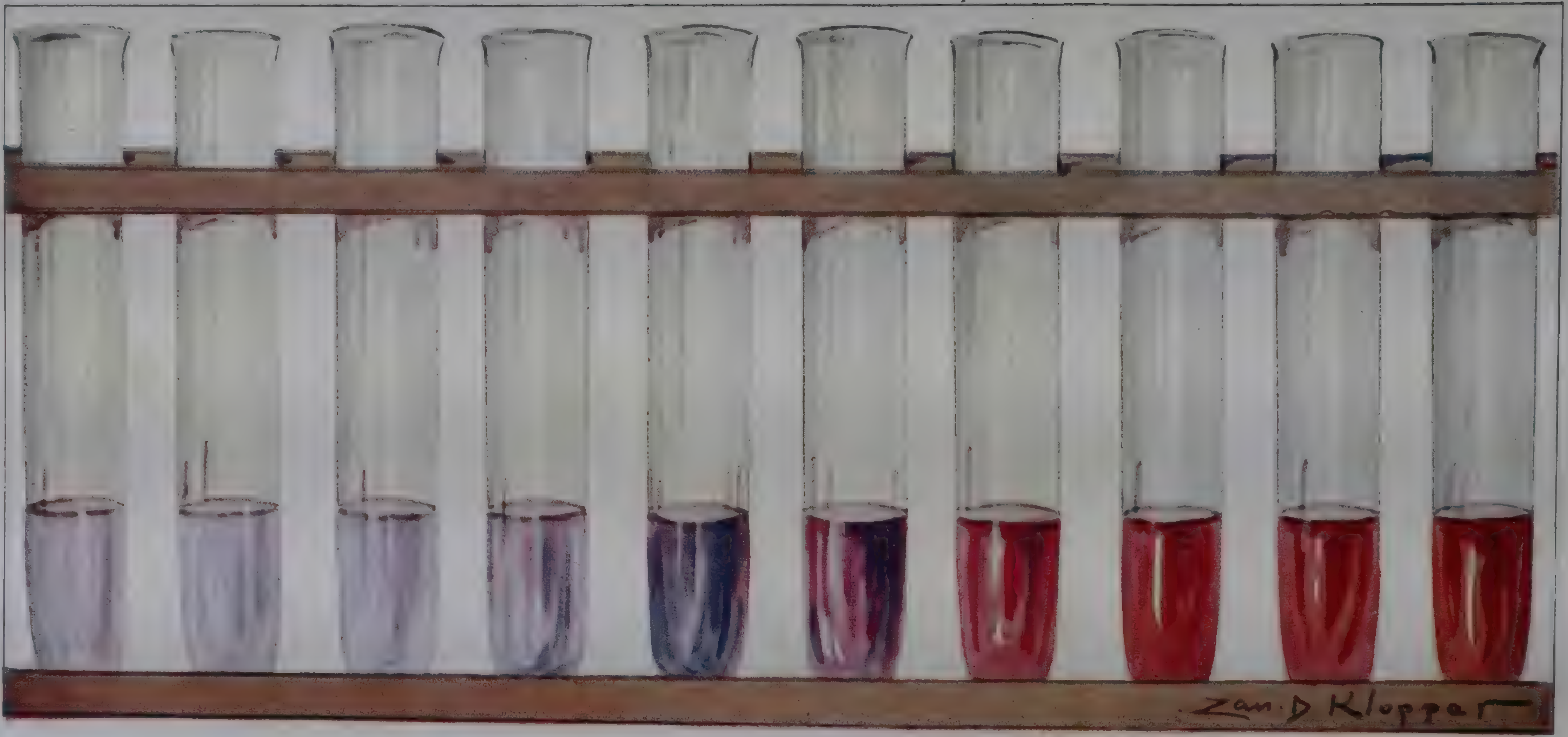

Plate XXX.—Colloidal Gold Reaction in a Case of General Paresis.
(From Levinson: Cerebrospinal Fluid, The C. V. Mosby Co.)

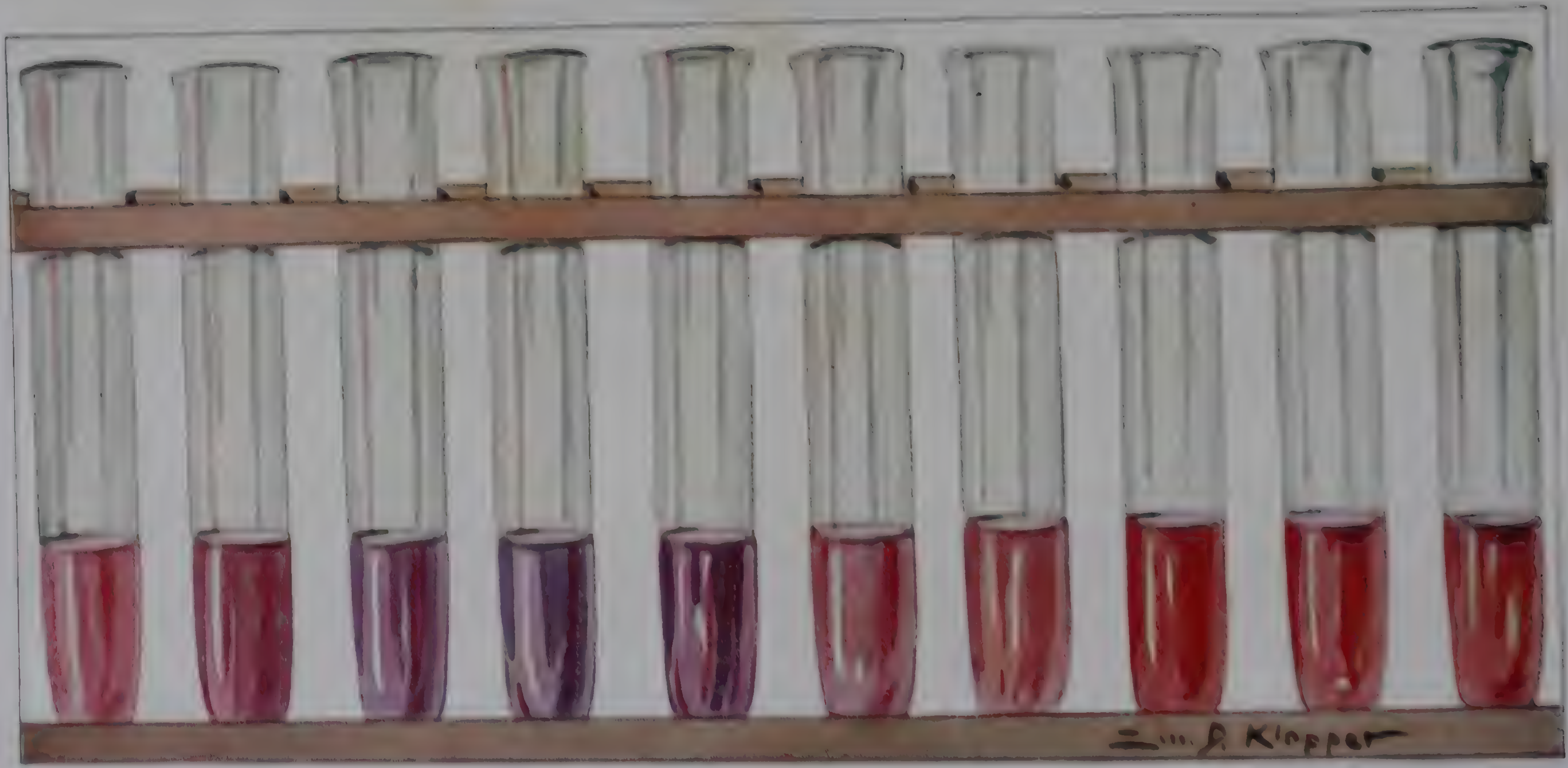

PLATE XXXI. COLLOIDAL GOLD REACTION IN A CASE OF TABES DORSALIS

(From Levinson: Cerebrospinal Fluid, The C. V. Mosby Co.)

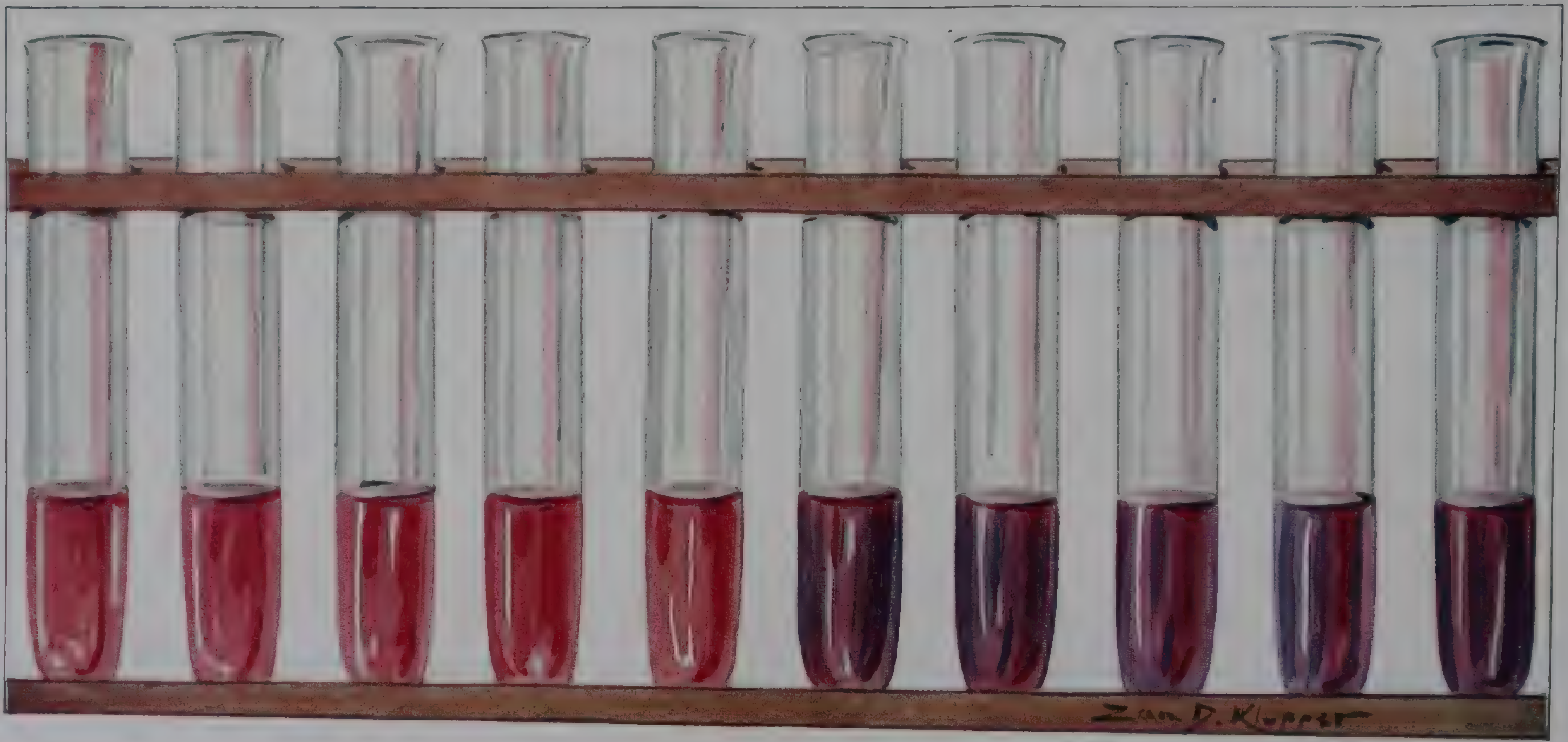

Plate XXXII.—Colloidal Gold Reaction in a Case of Meningococcus Meningitis.

(From Levinson: Cerebrospinal Fluid, The C. V. Mosby Co.)

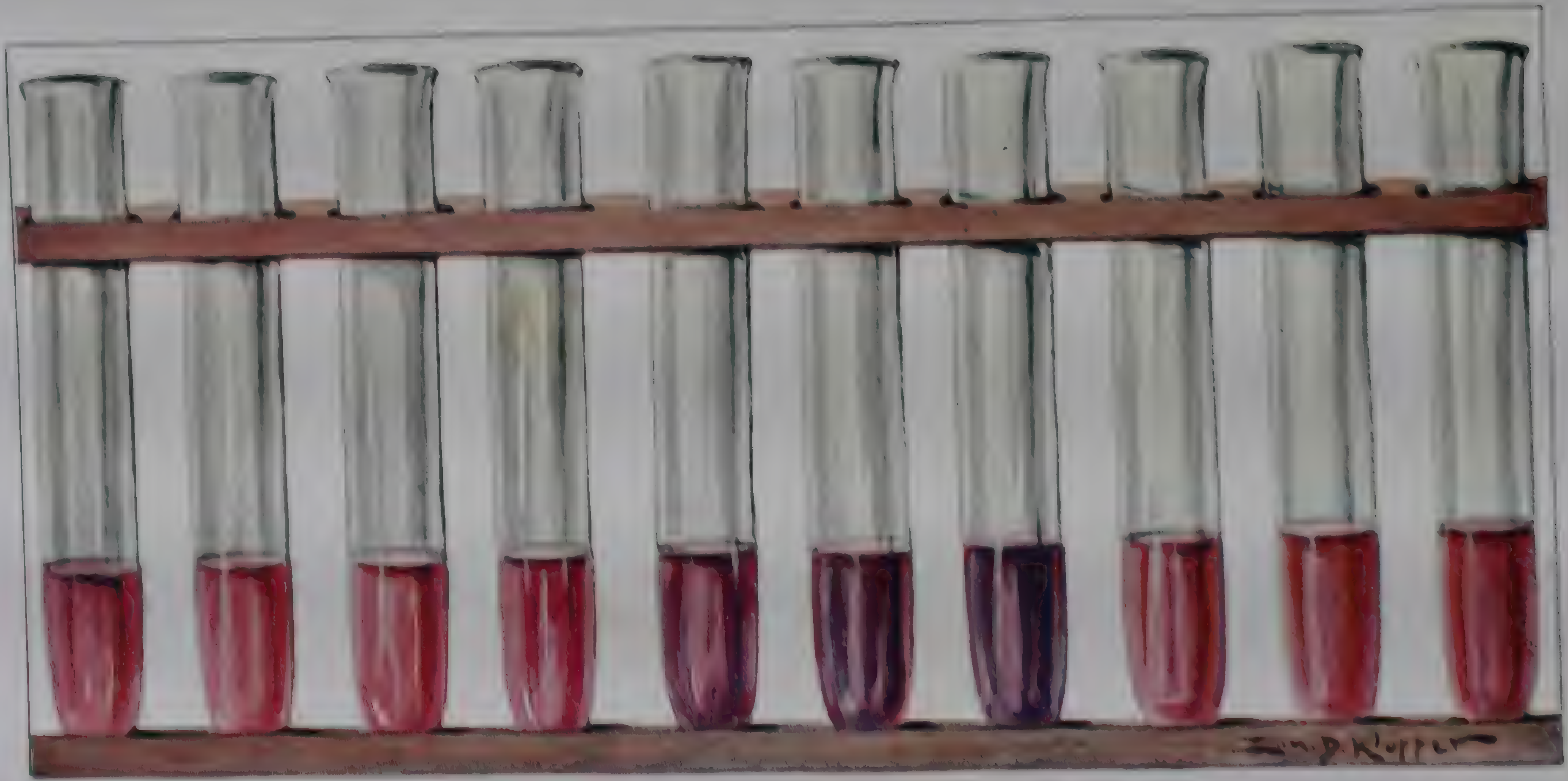

PLATE XXXIII. COLLOIDAL GOLD REACTION IN A CASE OF TUBERCULOUS MENINGITIS.
(From Levinson: Cerebrospinal Fluid, The C. V. Mosby Co.)

Glassware.—

1—1000 c.c. Pyrex beaker, free from scratches.
1—1000 c.c. cylinder graduated in 10 c.c.
1—1000 c.c. brown glass-stoppered bottle for storing the reagent.
1—100 c.c. cylinder graduated in 1 c.c.
1—25 c.c. volumetric pipette.
1—10 c.c. pipette, graduated in 0.1 c.c.
2—1 c.c. pipettes, graduated in 0.01 c.c.
2—5 c.c. volumetric pipettes.
Several test tubes, kept especially for use in testing the reagent.
Eleven test tubes for the actual test, 1 cm. in diameter, and 10 cm. long.

Technic of the Test.—

Test tubes, 1 cm. in diameter and 10 cm. long, the same size as Wassermann test tubes, are needed for the test. In addition, two 1 c.c. pipettes, graduated in 0.1 c.c., and one 5 c.c. volumetric pipette are required.

Physiologic saline is diluted to double volume for the test, as a 0.4% sodium chloride solution is used.

Place the 11 test tubes in a Wassermann test tube rack.

Leave one space between the tenth and the eleventh tube. Tube 11 is a control over the glassware and the reagent. It receives no spinal fluid.

Place 1.8 c.c. of 0.4% sodium chloride solution in Tube 1 and 1.0 c.c. in each of the remaining tubes, including Tube 11.

Add 0.2 c.c. spinal fluid, free from blood cells, to Tube 1.

Mix by drawing in and out of the pipette many times.

Withdraw 1 c.c. of the mixture in Tube 1, and place in Tube 2. Mix the contents of Tube 2 in the same manner, withdraw 1 c.c. and place in Tube 3. Mix the contents of this tube; withdraw 1 c.c.; place in Tube 4. Continue in this manner through Tube 10.

After mixing the contents of Tube 10, withdraw 1 c.c. of the mixture and discard. Tube 11 receives no spinal fluid.

Place 5 c.c. Lange colloidal gold in each of the 11 tubes.

Mix thoroughly and allow to stand at room temperature for 24 hours. Record the results according to the charts on page 1254 or record the numbers represented by the color changes in each tube according to the charts on page 1254.

The color scale, with corresponding numbers, is as follows:

5	colorless
4	pale blue
3	blue
2	lilac or violet or purple
1	red blue or very slight change
0	red, no change in the color of the reagent

The **principle** upon which this test is based is as follows: if a cerebrospinal fluid is diluted in geometrical progression with a weak neutral electrolyte and five volumes of clinical colloidal gold are added to each successive solution, a series of precipitation colors may be obtained which when ascribed numerical values from 0 to 5, and the values plotted as ordinates against the dilutions as abscissae, will give a curve whose contour is characteristic for certain of the infectious diseases of the central nervous system.

The recording of the color values or ordinates is then plotted on coordinated paper against the dilutions or abscissae. Multiply the abscissae values by some convenient factor to elongate the curve formed by connecting the points in a continuous line. A convenient factor is 5/3.

The color of the colloidal gold is red. No change in the color of the reagent is, therefore, red, or ''0'' on the color scale. Complete precipitation of the gold is accompanied by a change in color of the reagent from red to colorless, the color changes between red and colorless depending on the amount of precipitation.

Interpretation of Results

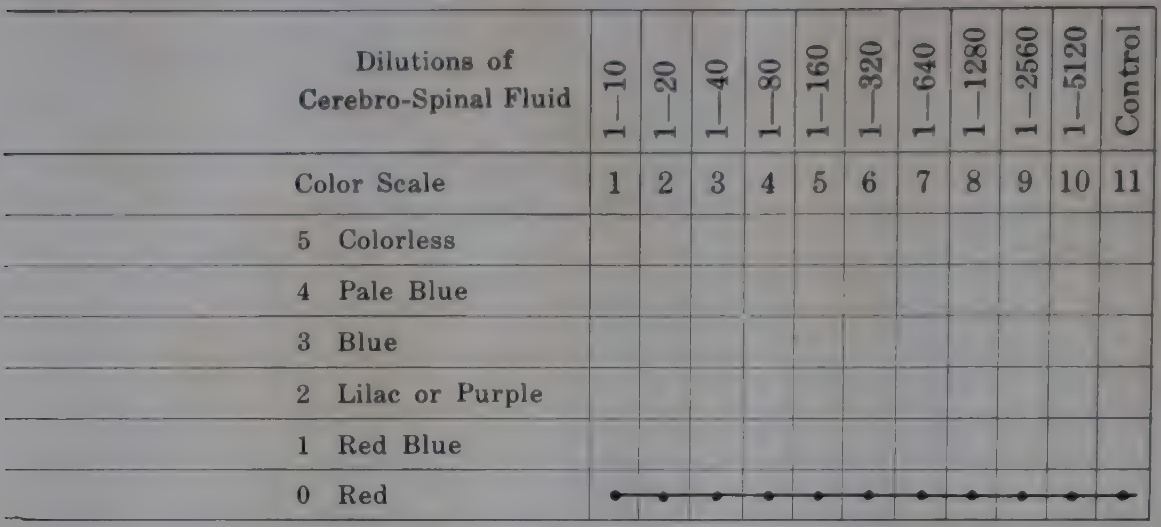

Chart I.—Normal curve 0000000000.

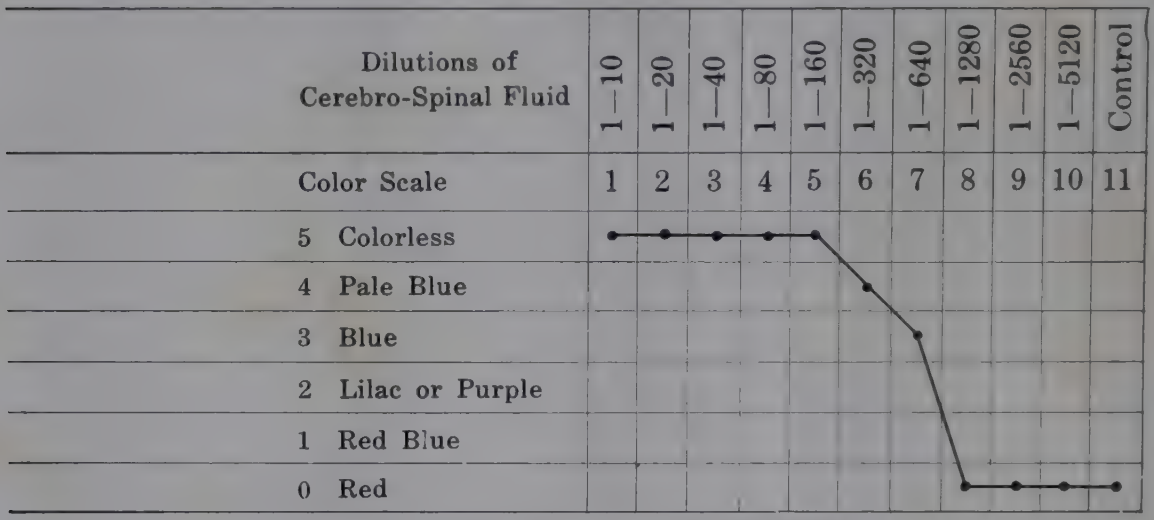

Chart II.—First-zone curve 5555543000.

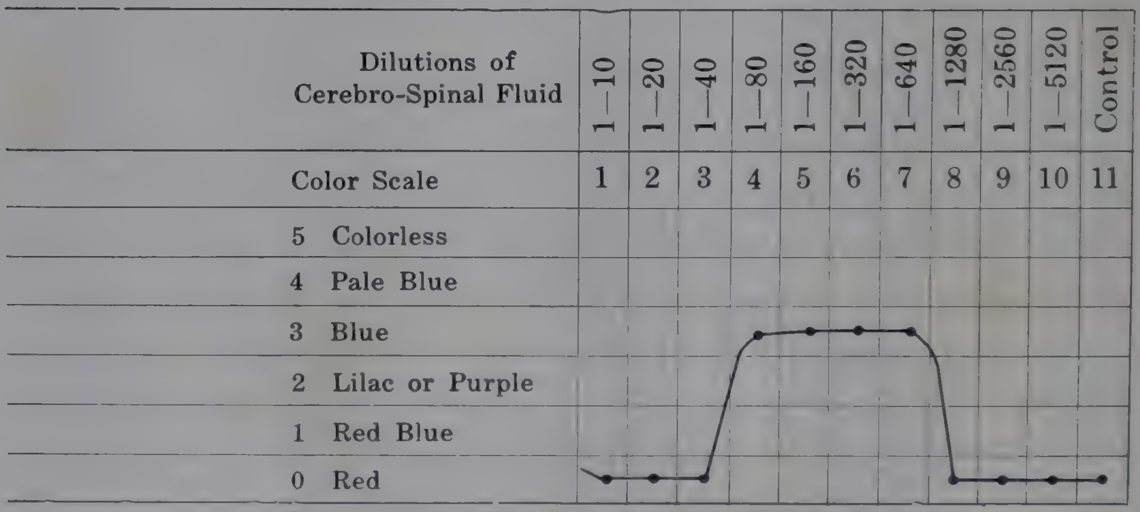

Chart III.—Mid-zone curve 0003333000.

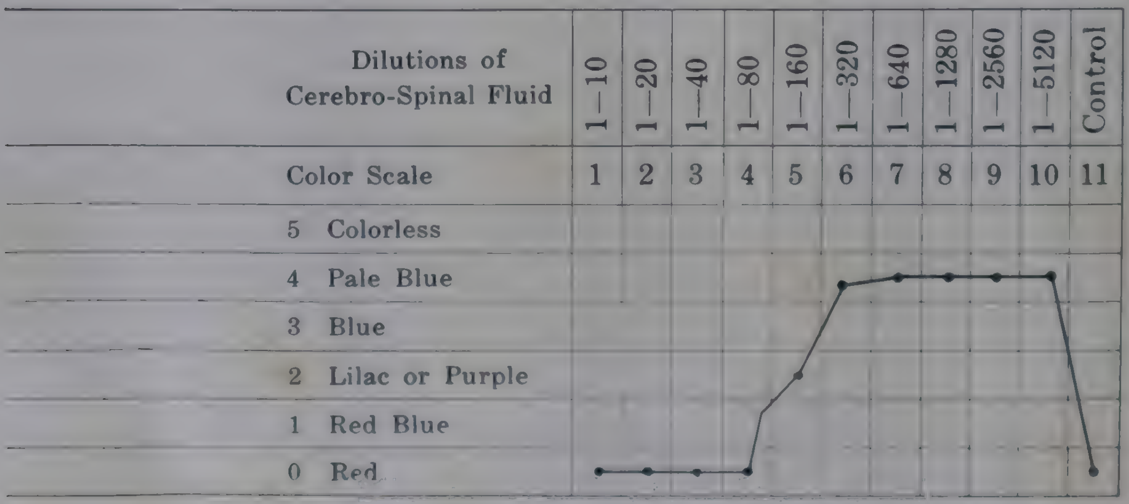

Chart IV.—End-zone curve 0000244440.

Interpretation of the Lange Colloidal Gold Reaction

It has been generally believed, ever since Lange brought out this reaction in 1912, that it is diagnostic of syphilis of the nervous system and particularly that various types of neurosyphilis could be distinguished by changes in this reaction. The weight of opinion at the present time, however, is to discard the terms "paretic," "cerebrospinal luetic," and "meningitic curves" and replace them by terms which indicate the tubes in which the greatest precipitation of the colloidal suspension occurs—"first-zone," "mid-zone," and "end-zone." This test indicates an abnormality in the protein content of the cerebrospinal fluid and does not necessarily have any particular diagnostic value. Kafka[1] and others have shown that the changes produced in colloidal suspensions of spinal fluids are related to the albumin-globulin ratio and to the total protein content of the fluid. In other words, the globulin fraction of the protein of the cerebrospinal fluid is most active in the precipitation of the colloidal suspension, while the albumin fraction has a protective action; this protective action depends not only on the albumin-globulin ratio but also on the total amount of protein in the fluid. A fluid with a high protein content and a high albumin-globulin ratio would produce no change in the colloidal suspension, but dilutions of it would cause a precipitation.

A normal curve results in no change of the colloidal gold suspension except possibly a slight change to lilac.

The "first-zone" curve is seen in the cases of paresis but is not necessarily diagnostic of this condition because it has been seen in multiple sclerosis and other conditions. (See illustration curves, page 1254.)

The "mid-zone curve" (see illustration, page 1254) may be found in any abnormal fluid and cannot be used as a specific indication of any particular type of cerebrospinal syphilis.

The "end-zone" curve (see page 1254) was formerly called the "meningitic curve," but really means a fluid with a very high protein content with an especially high albumin-globulin ratio. It is naturally found in cases of acute purulent meningitis, subarachnoid block, and extravasation of blood in the subarachnoid space.

It is perfectly reasonable, therefore, to find changes in the colloidal gold test without any other signs of syphilis in the nervous system. Too much emphasis has been laid in the past on the specific value of this test alone as a differential diagnostic measure in cerebrospinal syphilis.

Precautions With the Lange Colloidal Gold Reaction.—It must be remembered that the colloidal gold solution contains only 1 part of gold in 20,000 parts of water, and therefore the presence of even a minute trace of contaminant may have a disastrous effect on the proper precipitation color. It was shown by Zsigmondy[2] that when 0.5 c.c. of a 10% solution of sodium chloride is added to 5 c.c. of a gold solution containing 0.25 mg. of gold, more than 0.0025 to 0.005 mg. of gelatin or 0.003 to 0.02 mg. of alkali albumin[3] will prevent the change from red to violet.

---

[1] Kafka, V.: Die Zerebrospinalflüssigkeit, Leipzig and Vienna, F. Deuticke, 1930.

[2] Ztschr. anal. Chem. 40: 697, 1902.

[3] Schulz and Zsigmondy: Beitr. chem. Physiol. u. Path. 8: 137, 1902.

Probably all the errors made in connection with this test are due either to inadvertent protection of the gold solution before or after use, or inadvertent precipitation afterward. One must be sure to prevent the intrusion of even traces of foreign disturbing substances into these solutions. The spinal fluid must be received into specially prepared test tubes which contain no traces of gelatin or albumin. Using the salt solution in this test, it is advisable to pour out the quantity which is going to be used from the stock bottle into a separate clean container, so that repeated insertion of the same pipette or different pipettes into the stock bottle can be avoided. The same precaution should be followed with the colloidal gold solution; namely, pour out about 60 c.c. of this material from the stock bottle and reject the surplus.

Attention must be called to the fact that the smallest amount of saliva flowing into the pipette and touching the gold solution will inhibit normal precipitation of the colloidal gold solution. It has been found, too, that the soluble sizing from toweling used to dry a beaker has pronounced protective powers.

In order to check the gold solution and the salt solution, the addition of 0.4 c.c. of a 1% salt solution to 5 c.c. of the gold solution should produce no precipitation after standing at room temperature eighteen hours, while the addition of 1.7 c.c. should produce complete precipitation of the gold in the same period of time. The addition of 1 c.c. of a 1% solution of sodium chloride to 5 c.c. of the gold solution should produce, upon standing overnight, a color change adjudged to be midway between a "1" and a "2" reading. The ultimate test, of course, is to use the spinal fluid from a known paretic, which will give the typical curve of the paretic zone. If the gold solution should exhibit evidence of protection, a fresh 0.4% salt solution must be prepared with which the gold solution must again be tested. If the same results are obtained, it is probable that the gold solution has become protected through contamination; if an improved result is obtained, contamination of the original salt solution by a protectant is indicated; on the other hand, if an undue tendency for the gold solution to precipitate is found, and an improvement is then noted with the fresh salt solution, the presence of a precipitant in the original salt solution may be inferred.

The Test of Spinal Fluid for Syphilis

See discussion in Chapter XIII, Serology.

Bacteriologic Examination of Spinal Fluid

Spinal fluid is examined for the presence of tubercle bacilli, meningococci, various pus-producing organisms, influenza bacilli, and trypanosomes, viruses, etc. Refer to page 1601.

Examination of Spinal Fluid for Tubercle Bacilli

(See page 1602 for further information.)

According to many writers, the spinal fluid in tuberculous meningitis is clear, is under increased pressure, has a high cell count with lymphocytes predominating, shows decreased sugar and chlorides; it contains increased globulin; and one should be able to demonstrate tubercle bacilli by the special method which will be later described. Levinson contends that different kinds of protein in the spinal fluid distinguish tuberculous from "suppurative" meningitis. He uses a chemical test to differentiate meningitis due to the tubercle bacillus from other types of meningitis. In "suppurative" (nontuberculous) meningitis sulphosalicylic acid gives a precipitate three times as great as mercuric chloride. According to Levinson's experiences, in tuberculous meningitis the bichloride precipitate is supposed to exceed that of the sulphosalicylic acid.

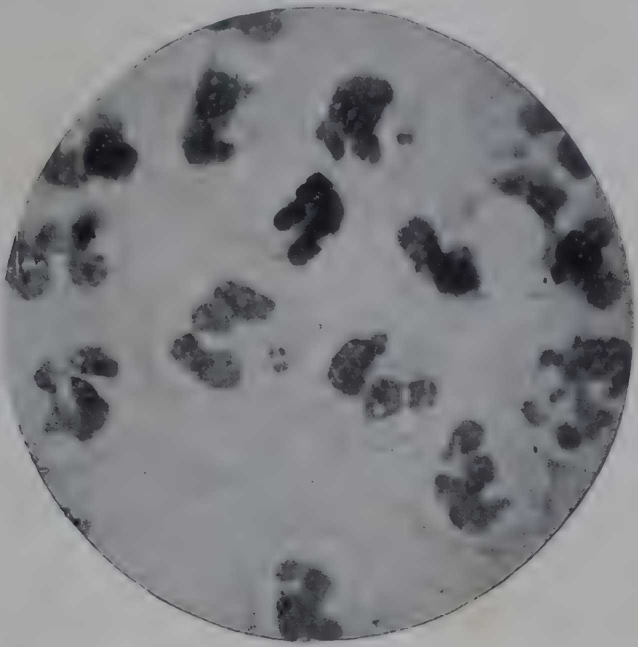

Fig. 305.—Photomicrograph showing direct smear from cerebrospinal fluid of case of meningococcus meningitis.

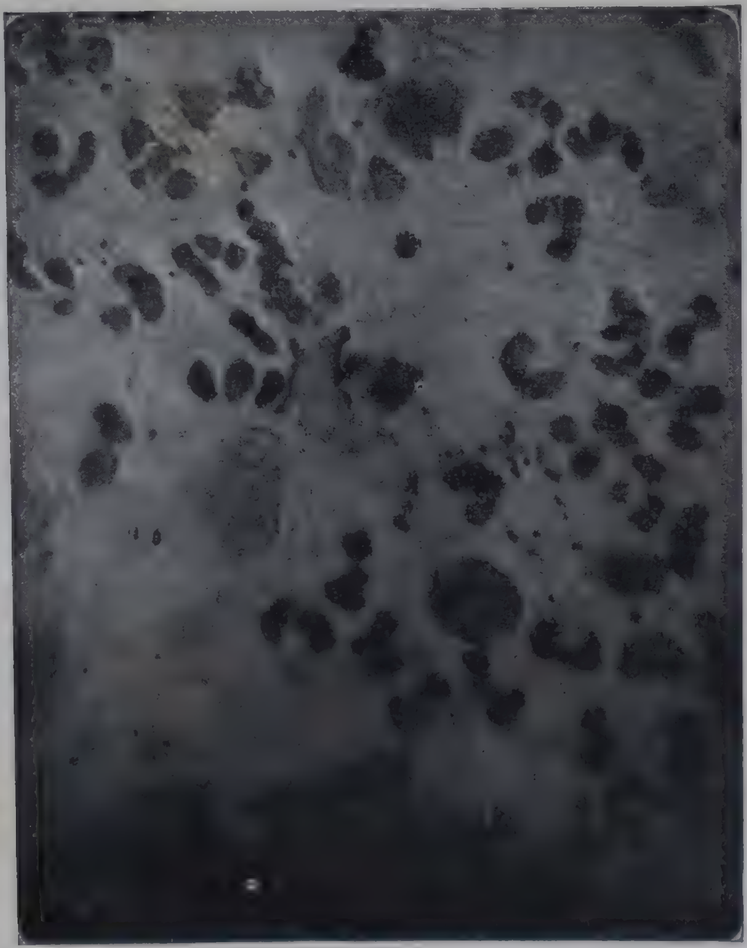

Fig. 306.—Direct smear of cerebrospinal fluid from another case of meningococcus meningitis. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.)

Some writers contend that the cells in spinal fluid of tuberculous meningitis are neutrophilic. Very few of the textbooks agree with Sahli[1] that the spinal fluid in unmixed tuberculous meningitis may be cloudy or turbid and contain an excess of neutrophiles. Traut[2] found in 113 proved cases of this condition, the spinal fluid was cloudy in 10% and neutrophiles constituted more than 50% of the cells in 10% of the cases.

In 17 of these cases, tubercle bacilli were isolated from the spinal fluid; the cases were also proved by autopsy. In 35 cases the diagnosis was made from the presence of tubercle bacilli in the spinal fluid. In 66 cases the diagnosis was confirmed by culturing tubercle bacilli from the spinal fluid on Herrold's medium,[3] and later by autopsy. In one case the tubercle bacilli were seen microscopically and cultured on Herrold's medium. In five cases the tubercle bacilli were seen and produced tuberculosis in guinea pigs. In three cases a positive guinea pig inoculation was the only proof. In one case tubercle bacilli were found only on guinea pig inoculation. This patient later showed tuberculous meningitis at autopsy. In all, autopsies were obtained in 76 cases. The records of 111 patients were obtained from the Cook County Hospital and the Presbyterian Hospital of Chicago. Two patients were in the West Suburban Hospital.

It is obvious, therefore, that one cannot expect to find clear spinal fluid in all cases of tuberculous meningitis, nor can one expect to find predominance of lymphocytes in all cases.

The examination of spinal fluid for tubercle bacilli is carried out as follows:

About 15 or 20 c.c. of spinal fluid should be withdrawn.

Direct Method.—

(1) Place 5 or 10 c.c. spinal fluid in a test tube. Centrifuge at high speed for ten minutes. Decant the supernatant fluid and discard. Smear the sediment on slides in egg albumen or serum. Allow to dry. Fix in the flame. Stain with Ziehl-Neelsen stain. Examine for tubercle bacilli.

(2) Incubate the spinal fluid for thirty minutes at 37° C. Fibrin threads will develop. Pick out the fibrin threads with a platinum loop, or centrifuge the specimen. Mount on slides in egg albumen or serum. Fix in the flame. Stain with the Ziehl-Neelsen stain. Examine for tubercle bacilli.

Cooke's Method.—

J. V. Cooke, of the Department of Pediatrics, Washington University School of Medicine and St. Louis Children's Hospital, has still a different method for demonstrating tubercle bacilli in spinal fluid. It is true that it is difficult to demonstrate tubercle bacilli because they are often present in very small numbers. It is probable that the thin fibrinous pellicle or clot which forms in these fluids enmeshes the bacteria and that stained specimens of this pellicle offer the best chance of finding the organisms. Cooke has found that the method he described facilitates the transfer of the fibrinous pellicle to the slide with a minimum of handling and distortion and has consequently helped to demonstrate tubercle bacilli. The test tube containing the spinal fluid with the pellicle is shaken gently to detach it from the sides or bottom of the tube, and the entire contents of the tube are poured on the surface of a clean glass plate, about 3 by 6 to 8 inches. Such plates may be made from discarded x-ray plates from which the emulsion has been removed.

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[1] Sahli, H.: Lehrbuch der klinischen Untersuchungs-Methoden, ed. 6, Leipzig, Franz Deuticke, vol. 2 (second half), 1920.

[2] Traut, E. F.: Am. J. M. Sc. **204**: 670, 1942.

[3] Herrold, R. D.: J. Infect. Dis. **48**: 236, 1931; **49**: 420, 1931.

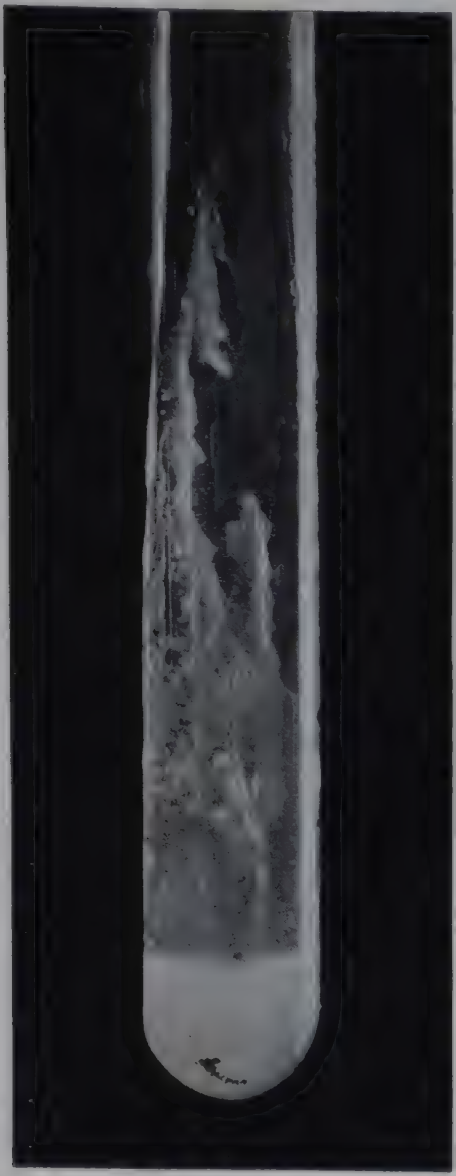

Fig. 307.

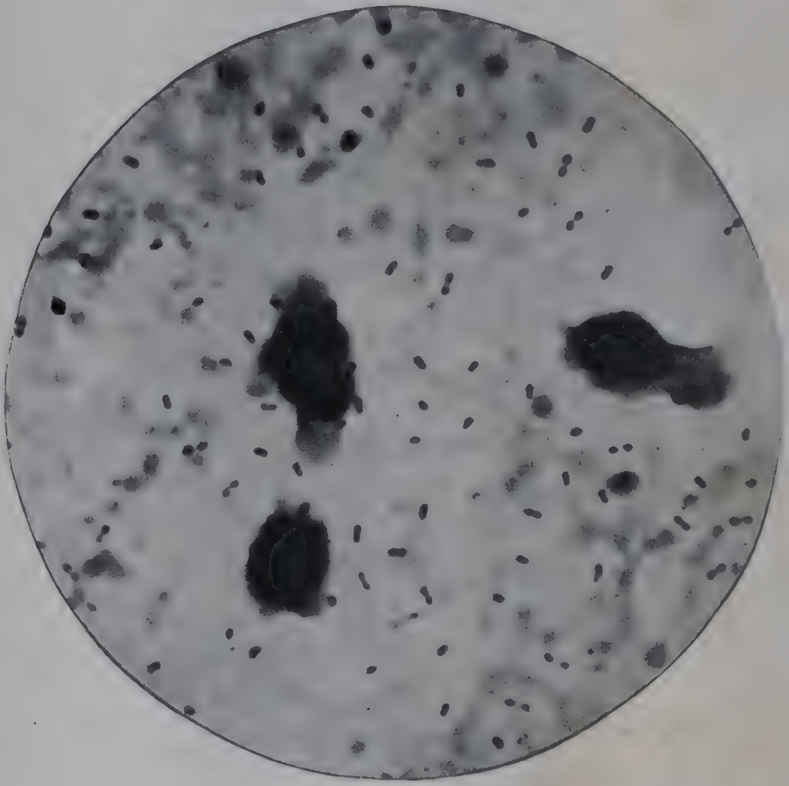

Fig. 308.

Fig. 307.—Twenty-four-hour culture of meningococci grown on ascitic dextrose agar. The culture which has been originally obtained from a case of meningococcus meningitis has been transplanted several times on artificial media, hence the heavy growth in twenty-four hours. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.)

Fig. 308.—Photomicrograph of smear from cerebrospinal fluid of pneumococcus meningitis. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929, The C. V. Mosby Co.)

Five to ten c.c. of the liquid will not over-run the edges of such a plate if moderate care is used in pouring it out. The fibrin film is not distorted and floats in the liquid on the flat surface of the plate. The excess of spinal fluid may be drained off with a pipette or blotting paper. After drying in the air, the film is stained in the usual manner (Ziehl-Neelsen method). Since the plate is too large to be satisfactorily placed under the microscope or to be satisfactorily used on the ordinary microscope stage, that portion bearing the stained film is cut down to 1 by 3 inches for observation.

Petroff's Method.—

To about 10 c.c. spinal fluid add 2 drops of 5% tannic acid solution. Shake thoroughly, until a precipitate forms. Centrifuge immediately at high speed for five minutes.

Decant and discard the supernatant fluid. Use one portion of the sediment for smears (made in egg albumen or serum); one portion for inoculation of appropriate medium (page 1647); and one portion for injection into guinea pigs.

Tests for Tuberculous Meningitis.—There are two special tests for tuberculous meningitis, the Levinson test and the tryptophane test. Neither of these tests is specific for tuberculosis but they are of some importance in an exclusion way.

Levinson Test.—

Place 1 c.c. of cerebrospinal fluid in each of two serologic tubes of equal size. Add 1 c.c. of 2 per cent aqueous mercuric chloride solution to the first tube, and 1 c.c. of 3 per cent aqueous sulphosalicylic acid to the second tube. Stopper tubes. Mix solutions and allow to stand upright at room temperature for 24 hours. Measure the height of the sediment in the two tubes and compare.

Normally, the sediment in both tubes is less than 2 mm. If there is an increase in proteins, the sediment in both tubes is heavy. The sediment in the mercuric chloride tube is light and feathery and forms slowly. The sediment in the sulphosalicylic acid tube is compact and heavy and forms rapidly.

It is claimed by the advocates of this test that in tuberculous meningitis the height of the sediment in the mercuric chloride tube is 2 mm. or more, and is two or more times that in the sulphosalicylic acid tube. In suppurative meningitis the sediment in the sulphosalicylic acid tube is greater than in the mercuric chloride tube.

Tryptophan Test.—

A positive reaction with this test occurs in bloody fluids, in purulent and xanthochromic fluids, whatever the cause; hence the test has no value in fluids of this character. In clear cerebrospinal fluid, the purple ring is demonstrable in most of the cases of tuberculous meningitis but the reaction is negative in encephalitis, poliomyelitis, brain tumor, and syphilitic meningitis. This is an extremely delicate test and its interpretation should be carried out very guardedly.

Technic.—

Place 2 to 3 c.c. of spinal fluid in a medium-sized test tube. Add 15 c.c. of concentrated hydrochloric acid and 2 to 3 drops of 2 per cent formaldehyde. Shake mixture and allow to stand for 5 minutes. Add 3 c.c. of 0.06 per cent aqueous sodium nitr*ite* by overlaying.

A negative reaction is characterized by absence of color change at the junction of the two fluids or by a faint yellowish color. A positive reaction is shown by a delicate violet ring which may take several minutes to become visible and which persists for fifteen minutes or longer.

Chick Embryo in Examination of Spinal Fluid

Refer to pages 1473 ff.

Trypanosomes in Spinal Fluid

Spinal fluid examinations are much used in tropical medical practice for diagnostic and prognostic purposes. Trypanosomiasis shows a high cell count in the spinal fluid indicative of the onset of nervous symptoms. The trypanosomes may be readily identified in the spinal fluid by routine smear of the sediment obtained by centrifugalization. The sediment is stained with Giemsa stain after preliminary fixation for five minutes in methyl alcohol. (See Plates XLVII and XLVIII.)

CHAPTER VIII

FECES

GENERAL CONSIDERATIONS

The examination of the feces, while generally stressed by clinical teachers as a very important means of diagnosis in medical and surgical cases, is seldom thoroughly performed. The leading gastroenterologists are undoubtedly examining stools, just as they are giving barium enemas, and making roentgenologic examinations of the canal, but the rank and file of the profession has not fallen into the practice. We do not, by any means, insist that there should be a routine chemical, bacteriologic, and microscopic examination of the stools of every patient who consults his physician, but we do believe that, in certain selected cases, a review of the stools will aid the examiner considerably. The microscopic examination can be quickly made, likewise the more important chemical tests; to wit, the search for occult blood by the benzidine test, and the examination for bilirubin and hydrobilirubin by Schmidt's method. A little time devoted to washing the stools in looking for gallstones will repay the effort. A search for pathogenic amebas is frequently indicated.

Physicians make exhaustive examinations of all parts of the patient but the anus and rectum; in most cases this part of the physical examination is superficial, and consequently much valuable information is overlooked. The indifference to the possibilities of help from routine stool examinations, lack of concise information as to technic and interpretation, and esthetic objection to handling the material, all have conspired to make this field of laboratory work practically a *terra incognita* to most workers and students.

In southern states we find more interest and more real work being done on stools than in any other section of the country, because of the prevalence of intestinal parasitism in that district and the impossibility of making a diagnosis definitely in any other way. We find a growing interest in stools, too, on the part of pediatrists, but in the main, we believe much more emphasis must be placed on this department than has been in the past.

NORMAL FINDINGS

It must be remembered that what constitutes the "normal" stool is quite a variable factor. There is no normal standard regarding either the frequency or absolute character of the stools; many factors enter into a consideration of what shall be the number, the character, the weight, the consistency, and the color of the passages; these factors must be accurately ascertained in every individual case before one can draw reasonable and proper deductions therefrom.

In order to appraise the individual case, certain fundamentals must not be overlooked. The feces consist of the indigestible and undigested remnants of food, plus certain constituents such as material secreted by the intestinal wall, plus certain bacteria which may or may not belong to the group of normal intestinal flora. The food remnants, together with intestinal parasites and their eggs, are intimately mixed with bacteria and excretions from the intestinal mucosa. It is claimed by Strassburger that ordinarily the feces consist of one-third bacteria; in pathologic conditions, this percentage may be markedly increased. There are many factors in the normal metabolism and habits of the individual that will determine the character and amount of the stool. Therefore, in summing up the data from this standpoint, the individual must always be considered. In some individuals one stool a day might be considered diarrhea; in others, constipation; in still others, normal. Diarrhea is usually considered to be an increase in the individual's frequency of evacuations; these may be from one to fifty a day.

The usual amount of stool is from 100 to 200 grams a day, but this quantity is dependent upon the amount and character of food ingested. Besides the diet, the amount of feces passed depends upon the adaptability of food to the peculiarities of the individual's digestion, the rapidity of intestinal absorption, the amount and character of the digestive juices, such as the bile and pancreatic fluids, the amount of mucus discharged by the intestinal mucosa, and the biologic characteristics of the bacterial flora of the canal. The age of the patient, his habits, whether he leads an active or a sedentary life, his occupation, etc., all influence the number and weight of the stools. Only an extended study of the individual will permit one to take these factors into serious consideration in an estimation of the status of intestinal activity.

Proper attention to the diet is necessary in computing the data from the weight of stools. Harley and Goodbody (The Chemical Investigation of Gastric and Intestinal Diseases, 1906) found that of a series of 118 cases on the same amount of mixed diet, the amount of the stool varied from 30 to 282 grams, an average of 102.8 grams. On a mixed diet weighing 150 grams, it is said that an individual ought to pass from 30 to 37 grams of fecal material, but owing to the factors already enumerated, this is by no means a constant figure. A diet rich in vegetable and starchy elements will increase the weight of feces, while one made up largely of protein, such as meat, tends to decrease this quantity.

The stools of patients with diarrhea lasting several days show undigested muscle tissue if they have been eating meat, also unchanged connective tissue elements. The predominant ingredient of a stool that is associated with inflammation of the small intestine is mucus, thoroughly mixed with food remnants.

Collection of the Specimen*

In order to make the best possible examination of the stool, the material must be properly collected and submitted to the analyst as rapidly as possible. This is particularly true with respect to ameba examinations, where, in order to detect their motility, the stool is examined, while still warm, with the aid of a warm stage. For routine bacteriologic and chemical examinations this is not vital. The best results for all purposes are obtained where conditions are such that no preservative need be added. For long distance transportation, a chemical preservative must be added; this sacrifices some of the data obtained from the fresh, unchanged specimen.

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*Refer to pages 1629 ff. for fuller details.

Physicians, as well as laymen, are prone to send both sputum and feces specimens in unwieldy, impracticable, and dangerous containers. The Post Office Regulations in this country very properly compel one to see to it that disease is not communicated through the medium of infectious material in the mails. Material is made safe for mailing if the proper bottles are selected, and if they are placed in double, tightly closed metal containers. The stool should be collected in a wide-mouth, chemically clean and sterile bottle. (We recommend throughout our work the sterilization of all glassware at 170° C. for two hours in the dry heat sterilizer.) The container must be chosen with the idea in mind of exactitude in diagnosis; first, to keep the material in much the same condition as it was when passed from the patient; second, to withstand the violence to which it may be subjected in transit. Despite reasonable care in transit, many very severe strains are placed upon such containers; they must be carefully packed so as to withstand the severest of ordeals in shipment. Sometimes the delay caused by the breaking of a specimen in transit may offset the necessity of an examination; at other times, the patient or examining or referring physician refuses to submit another specimen.

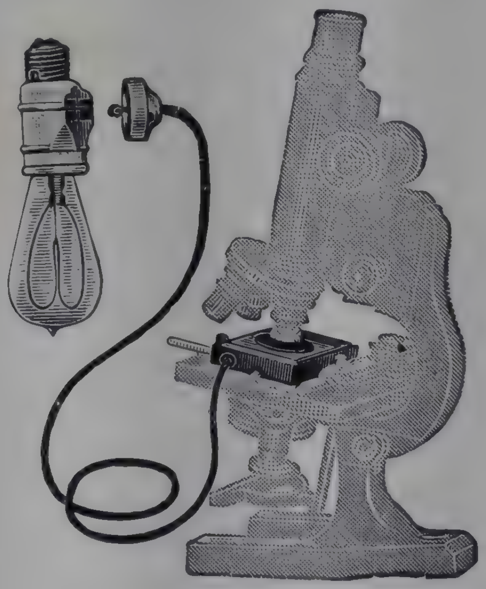

Fig. 309.—Warm stage. (Courtesy E. H. Sargent Co., Chicago.)

The receptacle and its packing must be so prepared that it will be neither a menace to the health of those handling it, nor offensive to the sensibilities of those who must examine it. Containers leaking fecal material from all sides, as from a Mason jar, for instance, require great care and time on the part of the laboratory worker to prevent infection and contamination of laboratory material. Above all, paper receptacles are to be condemned and never used.

Fill the bottle only about one-third full so as to allow ample space between the stopper and the top of the fecal contents to prevent the stopper from being forced out by gas formation. The stopper should be of cork and should fit tightly enough to prevent leakage. It should be long enough to project well above the bottle so as to facilitate ease of removal. It is well to tape the top of the bottle with adhesive strips after it has been closed.

The use of 20 per cent glycerin in physiologic saline solution has been suggested by Cumming who claims that it has an inhibitory effect on the growth of the colon bacillus and other fecal microorganisms. His solution is made by adding 20 c.c. glycerin to 80 c.c. of 0.9 per cent sodium chloride. To 20 c.c. of this solution, a mass of feces about the size of a split pea is added. Shake well to make uniform suspension. Do not use a greater amount of feces as the inhibition of *Escherichia coli* may not be controlled when larger amounts are used. According to Hazeltine[1] this method gives admirable results on specimens artificially contaminated with laboratory cultures of *Salmonella typhosa* and should be applicable in practice.

When the stools are to be examined in a hospital laboratory adjacent to the hospital-domiciled patient, they may be received directly into a clean sterile granite-ware bowl and taken immediately to the laboratory.

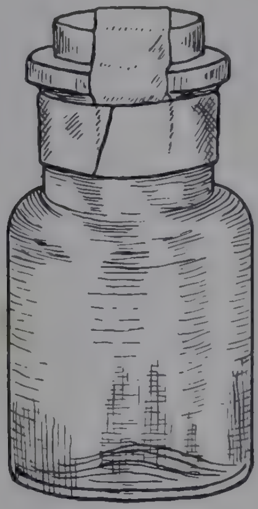

Fig. 310.—Taped bottle for collecting specimen of feces.

Stools intended for the demonstration of living amebas are examined while still warm. Preferably send the patient to the laboratory and procure and examine the stool there at once. Patients with dysentery due to pathogenic amebas may show these parasites only during the activity of the process when the patient has frequent bloody or purulent passages. The material should be examined at once, using a warm stage. If the patient cannot come to the laboratory, or the laboratory worker cannot take his microscope and other material to the bedside, then it is best to have the attending physician make half a dozen slides from the fecal matter, allow them to dry, and ship them to the laboratory. An examination of the stained specimen can be made for cysts; this is the most reliable method of diagnosis. In cases in which an examination is being made of the feces for amebas in a fresh state, the bottle or receptacle containing them should be placed in the incubator and kept warm while the technician is adjusting the microscope and warm stage. Refer to Chapter XII for details relative to examination of stools for intestinal protozoa.

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[1] Hazeltine, H. E.: U. S. P. H. S. Reports 32: No. 48, 1917.

In general, feces should be examined as expeditiously as possible because of the rapid tissue and bacterial changes. The isolation of *Salmonella typhosa*, for instance, increases in difficulty in proportion to the age of the stool. A specimen that has been quickly examined for typhoid bacilli will often show a good culture, whereas the same stool kept for a while and then cultivated will show no growth of typhoid. Certain changes of decomposition take place in the stool; the overgrowth of one or more hardy microorganisms prevents the growth and recognition of *Salmonella typhosa* in the stools of suspected carriers. The administration of a cathartic increases the number of bacilli in the stools. It is good practice, therefore, to precede the search for typhoid bacilli in suspected carriers by the administration of a saline cathartic. Bacteriologic examination of stools is discussed in detail in Chapter X.

As Cammidge has pointed out, the feces of infants have generally to be scraped off diapers and are therefore contaminated more or less with material derived from dusting powders or ointments which have been applied to neighboring parts. This must be taken into account in the chemical and microscopic examinations of infants' stools. The feces should be free from urine, to prevent interference with the reaction, the ammonia content, chloride estimation, etc. With adults it is not difficult to prevent the contamination of stool with urine. In the case of infants, the nurse may prevent contamination by placing a large pad of absorbent cotton over the urethral orifice while collecting the stool for the analyst. A stool that is strongly alkaline or that has an ammoniacal odor with a well-marked reaction for chlorides can be instantly proved to be contaminated with urine because the stool never shows more than a bare trace of chlorides. The presence of urine can be quickly proved as follows:

Test for Urine in Stools.—

Make a concentrated watery extract of the stool with boiling distilled water. Filter. Acidify with nitric acid and add a 10% solution of silver nitrate, adding this drop by drop. An uncontaminated stool will show only a slight opalescence due to the formation of silver chloride. If a heavy white precipitate forms, indicating a large amount of chlorides, this stool contains urine.

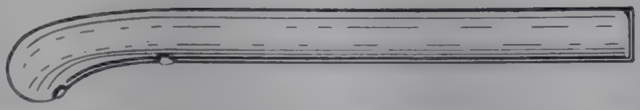

Fig. 311.—Appliance for removing stools from infants for bacteriologic examination.

In reference to stools intended for bacteriologic examination, as noted above, it is often advisable to give a sterile enema, plus a purgative, but this is not necessary or advisable when making a chemical analysis only. Stools intended for chemical analysis should not be contaminated by any oil or soap enemas. It is imperative for the analyst to know what drugs, if any, the patient has been taking; particularly, iron, bismuth, liquid paraffin, olive oil, etc. If these are being used, they should be discontinued a few days before the chemical analysis is undertaken.

The stool of an infant should be obtained for bacteriologic analysis by means of a special sterile feces collector directly from the lower end of the rectum. Such tubes have been devised by Cohnheim and Sato. This glass instrument, previously sterilized, is lubricated with sterile vaseline and introduced into the lower end of the canal, turned about, and a small amount of the stool caught in the eye of the instrument, then quickly withdrawn and the material examined bacteriologically.

Only a small amount of feces is necessary for the usual routine stool analysis. In cases in which an elaborate chemical analysis is desired, the stool must be collected for several days and a large quantity submitted. We do not recommend a complete chemical analysis of stools except under special conditions, where a complete metabolic study of the individual is being made. For instance, to determine the metabolic changes in a given case, we might make such a chemical analysis in conjunction with the estimation of the chemical intake of food, the study of the blood chemistry of the individual, and the chemical study of the urinary constituents. As a rule, the stool examination should consist of a determination of certain essential factors, macroscopic and microscopic. Under special conditions this examination may be varied to include a complete bacteriologic study, qualitative and quantitative.

Test Meals

In order to test the income and outgo of nitrogen and other elements, it is customary in food and metabolism experiments to put the patient upon a certain definite quantity of food, together with the ingestion of some substance at the beginning and at the end of the experiment which differs enough in color and consistency from the surrounding feces to render easy the differentiation of the feces of the ''experimental period'' from that immediately preceding and succeeding it. A method which is very serviceable is to give a gelatin capsule containing 0.2 gm. of powdered *charcoal* at the beginning and end of the period under observation. This causes the appearance of two black zones to map off the intermediate period of nutrition which we are engaged in studying. *Carmine* in 0.3 gm. capsule may also be used in the same way, thus producing two dark red zones.

The test meal of Schmidt[1] is possibly the most useful:

> One liter of milk, 100 grams of zwieback, 2 eggs, 50 grams butter, 125 grams beef, 190 grams potato, 1 gram oatmeal, sodium chloride 2 to 3 grams. This contains 102 grams albumin, 111 grams fat and 191 grams carbohydrates, representing 2,234 calories. The material may be divided as follows:
>
> *Breakfast*—0.5 liter milk, 50 grams zwieback, 0.5 liter oatmeal. Gruel made of 40 grams meal, 10 grams butter, 200 grams milk, 300 grams water, and one egg, and enough salt to make it palatable.
>
> *Luncheon*—125 grams chopped beef (Hamburg steak) with 20 grams butter lightly baked so that it is practically raw, plus 250 grams potato soup made of 190 grams mashed potato, 100 grams milk, 10 grams butter, and salt.
>
> *Afternoon Luncheon.*—0.5 gram milk, 60 grams zwieback.
>
> *Evening Meal.*—0.5 gram of morning oatmeal, gruel, with the same amount of butter, milk, water, and egg and salt.

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[1] Die Funkstionprufe des Darmes mittels der Probekost, ed. 2, Wiesbaden, J. F. Bergmann, 1908.

This diet is given for three days or longer, until one can be assured that the patient is passing a stool composed of the remnants of this food. The test meal produces a normal stool which can be identified by giving 0.3 gm. carmine to color the stool at the beginning of the transition from the normal diet. If the milk produces diarrhea, cocoa may be substituted in the same quantity, made by mixing 20 gm. cocoa, 10 gm. sugar, 100 gm. milk, and 400 gm. water.

EXAMINATION OF STOOLS

The examination of stools is divided into five studies: first, macroscopic; second, microscopic; third, microchemical; fourth, chemical; and fifth, bacteriologic.

Macroscopic Examination

The macroscopic identification of parasites may be summed up as follows: the proglottids of the tapeworms, *Ascaris lumbricoides, Enterobius (Oxyuris) vermicularis* all are likely to appear spontaneously in the stools. *Ancylostoma duodenale* and *Trichuris trichiura* appear only as a result of definite therapeutic attempts to expel them. Rarely we find insects and their larvae. I recall a case near St. Joseph, Missouri, where the stools repeatedly showed the larvae of flies, and where the physician and patient both stoutly maintained that all chance of contamination after the stools were expelled had been excluded. We were never able to prove this. Workers must be sure that stools are not contaminated *after* their expulsion either accidentally or purposely, by malingerers or psychopathic individuals.

The number and variety of foreign bodies that have been found in stools are legion. They may vary from meat skewers, splinters of wood, eggshells, garden earth (from imperfectly cleaned vegetables), kernels of nuts and fruits, pieces of tobacco leaf, tea leaves, etc. It is remarkable to note that sharp instruments may pass the entire length of the canal and may be expelled without injury to the wall, such as needles, pieces of a knife blade, glass balls. It must not be forgotten that some of these foreign bodies may be placed in the anus by psychopathic persons.

The macroscopic examination is very important.

Place a portion of the stool on a flat dish. Note the color, consistency, quantity, mixture with large mucous particles, blood, and undigested food remnants. For closer examination, some of the stool should be mixed with water and this diluted material should be put in a Petri dish with a dark background. The brown particles which will then be noted should be examined microscopically.

Pathologic macroscopic findings are as follows:

1. Undigested food remnants; such as connective tissue, remnants of potatoes and meats, and pieces of fat.
2. Mucus, pus, or blood.
3. Pieces of human tissue.
4. Intestinal stones and gallstones.
5. Worms.

The **Physical Characteristics** of the stool should be investigated as follows:

Color.—Under normal conditions the color of the stools may be light yellow to blackish blue, dependent in a measure upon the character of the food ingested. Exposure to the air and to light causes the stool to become blacker. The fecal color is due to bile pigment, plus the presence of foreign pigments of foods and drugs. The ingestion of blackberries or huckleberries causes black stools; chocolate gives them a dull gray color; cocoa makes them light gray. The effect of starchy food is to produce a yellow stool; of chlorophyl from vegetable material, a greenish shade. Fats produce the classical "clay-colored" stool. Certain drugs, such as bismuth and iron, produce a black stool; senna, rhubarb, santonin, and calomel give a yellowish green cast.

The normal color of the fecal material is due to the presence of hydrobilirubin derived from the bilirubin of bile; the intestinal bacteria convert bilirubin into hydrobilirubin. Neither of the bile pigments, bilirubin nor biliverdin, is ever normally encountered in a stool unless the bile is passed unchanged down the intestinal tube by unduly rapid peristalsis. The darkening of the stool after exposure to the air is probably due to the conversion by bacteria of hydrobilirubinogen into hydrobilirubin. The *color* of *stools* is mainly dependent upon the character of the *diet*. If one is to be influenced by color of the stool in estimating a fault in health, then he must inquire very carefully as to the diet taken, whether mixed, whether it contains vegetables or fruit or what not. An inquiry of this kind will usually explain the color.

Blood.—Blood is not easy to identify in the stool because the bright red color is lost after the blood remains some time in the intestine, changing in color to the brown of hematin. A large quantity of blood gives the appearance of cooked tea. If blood comes from the lower bowel, it will usually appear on the surface of the stool and can be detected by the naked eye. It is always red if it comes from the rectum and is darker if it comes from a point higher up. When summing up the evidence presented by either the macroscopic or the chemical analysis for blood in stools one must know whether there are any hemorrhoidal changes present, also whether there is a rectal fissure, as well as polyps, rectal adenomata, carcinoma, or ulcer. Attention should be called to the gross appearance of stools contaminated with large quantities of blood coming from points higher up, notably the "tarry" color of duodenal ulcer, typhoid stools with bleeding, or in severe dysenteric conditions.

Resort must be had to the microscope and the chemical test to identify blood.[1] Blood mixed with pus is seen in dysentery. Mucus and blood are seen mixed in cases of invagination and polyposis intestinalis. Blood is found, too, in cases of embolism of the mesenteric arteries. In simple catarrh, blood may be found just as it is found in so-called stagnation colitis (hemorrhoidal bleeding). Nothnagel noted bleeding in tuberculosis without ulcers and without catarrh.

Form.—The form of the fecal material gives an idea of certain conditions. The stools in prolonged constipation are hard and often ball-shaped, sometimes grooved by the intestinal bands. There is a characteristic scybala formation called "goat-droppings" seen in cases of spastic constipation or we may see pipe-stem or ribbon-shaped stools. In general it may be said that the formation of scybala, due to small bulk of feces and lack of moisture, is evidence of spastic constipation.

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[1] See pages 1290 ff. for occult blood tests.

Liquid and semisolid stools are indicative of irritation in the intestinal tract, all comprised under the general term of "diarrhea." There are varieties of these liquid formations, sometimes spoken of as characteristic of certain definite disease states, such as the "pea-soup" stools of early typhoid, and the brilliant red stool of the later period. In cholera the so-called "rice-water" stools are seen.

Odor.—The odor of feces is due to the presence of indol and skatol which are formed as a result of the putrefactive processes that normally occur in the intestine. These putrefactive processes result from the action of bacteria upon protein present in intestinal contents. The most important products formed are indol, skatol, paracresol, phenol, para-oxyphenyl-propionic acid, para-oxyphenylacetic acid, volatile fatty acid, hydrogen sulphide, methane, methylmercaptan, hydrogen, carbon dioxide, proteoses, peptones, peptides, ammonia, and amino acids. Of these products indol and skatol in the feces give the disagreeable odor, while indol, skatol, phenol, and paracresol appear in the urine as ethereal sulphuric acids. Methane, methylmercaptan, and hydrogen sulphide may add to the disagreeable odor produced by indol and skatol. The more meat digested by the patient, the more the odor; it is less marked in stools from a vegetable diet and hardly noticeable in stools from a milk diet. This explains the fact that stools of infants on milk diets are practically odorless except under pathologic conditions.

Casts.—Casts of the bowels are occasionally found. They may be passed in shreds, or in the form of a complete cast of the intestine. This condition has been designated by Nothnagel as "enteritis membranacea" or "colica mucosa." They may be a foot or more long and may be passed, accompanied by severe tenesmus, without any fecal material. These cases are seen more commonly in women than in men, and have been regarded as indicative of hysterical or nervous disorders. They are found according to Greene[1] associated with two conditions aside from the causes mentioned; namely, impairment of general nutrition, and spastic constipation. The nature of these casts may be determined by Pariser's method:

Treat them with sublimate alcohol and then with Ehrlich's triacid stain; they turn green if mucoid, whereas, if fibrinous, they turn red. Details of the test are given on page 1271.

Undigested food particles may be seen and identified by the naked eye. Pour liquid stools into Petri dishes. Thick pasty stools are best stirred with water and then poured over a fine shred sieve. The sieve described by Einhorn is very useful to detect concretions. Another method without the use of a special sieve consists of tying a double thickness of cheesecloth over a ring of wood or on to the edge of a pail. Kemp has described a method which is simple and practical. A circular wire is fitted to the toilet seat and to this is attached a bag made of double thickness of cheesecloth which dips into the pail. The patient defecates into the bag. Water is poured through until only the more solid parts remain. These are then scraped off and examined. The same thing may be accomplished by means of a colander over which is laid a fine meshed wire screening.

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[1] Medical Diagnosis, Philadelphia, 1926, P. Blakiston's Son & Co., p. 865.

Undigested food particles, pieces of bone, cartilage, and muscle may be seen as undigested food remnants. Starchy foods leave no residue that can be distinguished by the naked eye, but raw vegetables such as cucumbers, lettuce, onions, radishes, asparagus, and string beans, and fruits, such as cranberries, nuts, and currants, pass through the intestinal canal and appear intact. It is difficult to recognize the residue of cooked vegetables unless they are very poorly masticated. Among other products which may be recognized with the naked eye are the epidermis of fowl, fish scales, pieces of lobster shell, or eggshell. The connective tissue and sarcolemma of "old hens" may be recognized also. The ingestion of considerable fat may be recognized in the stool, according to Nothnagel.

In general the process of cooking of food is reflected in the condition of the food remnants in the stools. There is a more thorough disintegration of meat fibers from long-continued stewing and boiling than there is from baking. Small pieces of brownish baked meat and even particles of fried eggs may be recognized by the naked eye.[1] The cooking of vegetables in "hard water" makes the cellulose tougher, so that there are distinct food remnants in the feces.

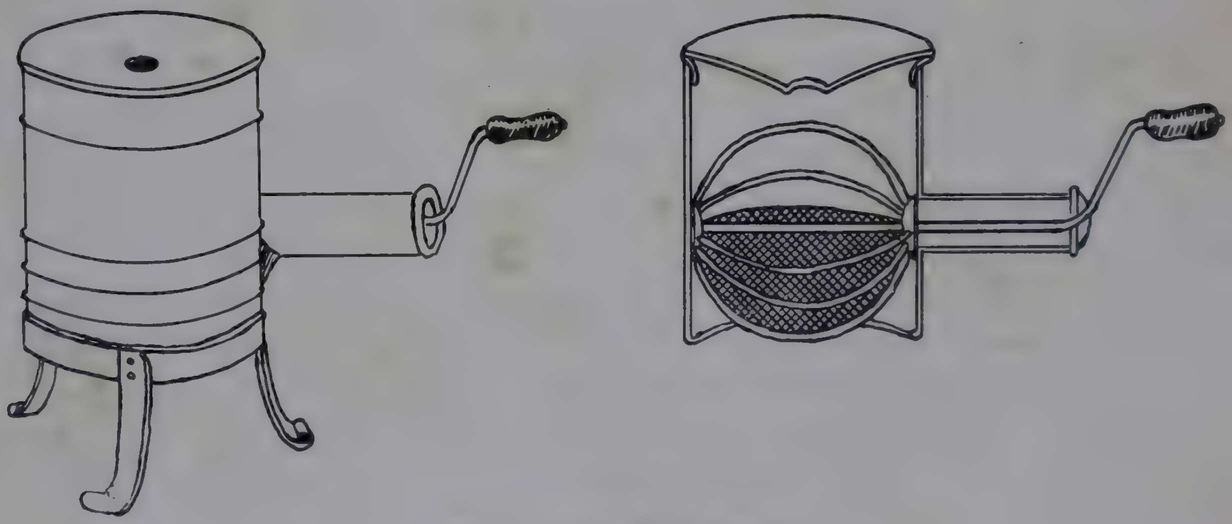

Fig. 312.—Einhorn sieve.

The factors of imperfect mastication of food and deficiency in the motor functions of the gastrointestinal tract are important in a consideration of food remnants in the stools. The influence of chemical insufficiency of the gastric and intestinal juices is also important. Ogata introduced raw meat in dogs directly into the duodenum and found the connective tissue of the meat in abundance in the stools. The same result is seen in cases of achylia gastrica. Schmidt noted in all cases where there was a deficiency of the gastric juices that connective tissue residue of meat predominated in the passages. Faber found in cases of achylia and hypoacidity that fish bones and small spicules of bone appeared constantly in the stools, but not so in health. Insufficiency of the pancreatic function is seen in albuminous and fatty changes in the stools; masses of fluid fat appear, which become hard when the stool is exposed to the air, so that it becomes covered with a fatty crust. Deficiency of the biliary function also causes a disturbance in fat digestion, with much fat in the stool. Deficiency in the resorption powers of the gastrointestinal tract is also responsible for food remnants in stools. Schmidt reported a case of tabes mesenterica with visible muscle remnants in the stool.

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[1] Van Ledden-Hulsebosch: Macro u. mikroscopische Diagnostik der menschlichen Excremente, Berlin, J. Springer, 1899

The entire question of food remnants in the stool, from a diagnostic standpoint, must revolve around the determination of what is normal or abnormal for the particular individual. Confusion often exists in the minds of beginners in examination of stools; they often mistake one kind of food remnants for another, and for parasites. The microscope must be constantly at hand to correct such errors. Small pieces of muscle may be mistaken for bile-stained fat and vegetable residue, or even clumps of mucus. Pieces of orange skin have been mistaken for parasites. The pieces of mucus found in enteritis membranacea have been mistaken for actual pieces of intestinal mucosa. We should search, especially in the stools of infants, for white masses formed from milk. They occur in gastrointestinal disturbances in infants and are formed from casein, according to Monti; often from fat, according to Widerhofer.

Referring to the test diet (see page 1266) the following directions may be formulated: if there are particles of chopped beef in the stool there is derangement in gastrointestinal digestion. If connective tissue remnants are seen, the stomach is at fault. If muscle particles are visible and no connective tissue, it is an intestinal condition, probably a fault in pancreatic juice digestion. If both connective tissue and muscle remnants are seen, then both stomach and intestine are involved. We term a condition with undigested visible food remnants "lientery."

The determination of the state of gastrointestinal activity from the presence of undigested *vegetable remnants* is much more difficult than is the case with meat. Ledden-Hulsebosch stated that in health, food made of flour, white bread or potato leaves no residue visible in the stool, but that raw vegetables such as radishes, cole slaw (cabbage), pickles, onions, etc., pass out unchanged. The hard vegetables such as asparagus, rhubarb, string beans, as well as poorly cooked or poorly cut up beans, peas, lentils, and the like, are readily seen in properly functioning gastrointestinal tracts. Bean soup, spinach, and the like are unrecognizable. Fruits, such as berries, currants, etc., nuts, are unchanged, as well as the skins of plums and apples. Under a test diet, cooked vegetables are not to be seen in the stools. The cellulose fibers of vegetables may be seen in a general diet, but no data can be drawn from their appearance.

Mucus.—Mucus that appears in the stool is usually visible macroscopically. In very small quantity it is difficult to find, but in larger quantity it can readily be seen and grasped with the forceps. Cases in which it is found only with the aid of a microscope are rare, but do exist. Our interest, therefore, is in mucus that can be found macroscopically.

Tests for mucus may be made as follows: A few particles of the supposed mucus are washed in water and stored in a test tube with 2½ per cent mercuric chloride in alcohol, using a glass rod. The larger particles are thrown down by the hardening effect of the mercuric chloride in alcohol. Sediment the specimen, add distilled water with a few drops

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of Ehrlich's triacid stain, sediment again, wash in water, and examine microscopically. The mucus particles, provided they are not too rich in cells or fat, are stained bluish green or green; all other animal cells are stained red.

Mucus is precipitated with acetic acid. The precipitate is not redissolved by an excess of this acid. Mineral acids added slowly cause a precipitate; added rapidly they dissolve the mucus. Alkalies cause it to swell and dissolve. Alcohol causes shrinkage. Thionin stains mucus violet, other tissue remains blue. In order best to obtain this reaction, take care that there is not too much mixture with other material, and that the reaction of the specimen is neutral. Other aniline colors stain only the cells present. Iodine stains a diffuse yellow color. Hecht's method[1] is a mixture of brilliant green (2 per cent) with neutral red (1 per cent). Stain a few minutes. This gives a light red color to mucus, which is in sharp contrast to the rest of the field, which is green. Nuclei and cell membranes appear reddish violet.

The amount of mucus present in stools may range from very small particles to enormous masses. We have seen pieces of mucus in cases of membranous colitis as long as 40 cm.

The mucus is usually present in clumps or small masses which, in water, appear to have irregular edges. At times there are the so-called boiled sago nodules, the composition of which has been much in dispute. The consistency of mucus masses varies from extreme softness to leatherlike hardness. Pure mucus masses are usually soft, the harder particles consisting of other material as well as mucus; namely, albumin, fat, and many cells. Mucus is transparent, but when contaminated with other substances, such as fat or albuminous material, it becomes cloudy. It imitates the color of the fecal mass in which it is found, varying from the brown color of hydrobilirubin to the orange red and green color of bilirubin and biliverdin; blood elements, of course, make mucus reddish or red brown.

Washing the stool gently with water makes it comparatively easy to find the small microscopic particles where the macroscopic view fails to reveal them. Mucus appears under the microscope as structureless material, more or less transparent, in which may be imbedded epithelia, pus cells, red blood cells, bacteria, protozoa, air bubbles, detritus, food remnants, and crystals. Mucus from the upper tract is more likely to be entwined with food residue than when it is from a point low down. The bacterial contents vary, being very scant from cases of mucous colitis. The greater the transparency of the mucus, the less the contaminated bodies bound up with it. Note two points of interest, the irregular lines that traverse it, and the irregularity of its boundaries.

Only visible mucus is found under pathologic conditions. The exceptions to this are the meconium and the stools of young infants. Caramer[2] noted the presence of mucus in the first meconium of newborn children. In constipated individuals, the hard stools literally scrape off particles of the mucosa.

The bodies described by Nothnagel, which he terms "yellow mucous granules" or hyaline mucous islands, are not mucous in character, but are of an albuminous or fatty nature. They are minute pinhead particles which may be present in very large numbers, yellowish brown, and soft. Pressed under the microscope, they spread out and are homogeneous, free from cells or other structures, thereby differing from mucus which is frequently combined with cellular material. They originate high up and, being bile stained, certainly indicate an ileitis or jejunitis, as claimed by Nothnagel.

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[1] Wien. klin. Wchnschr., 1908, No. 45.

[2] Deutsch. med. Wchnschr., 1907, No. 12.

The most important consideration in the study of mucus is its *source*. Usually, *mucus comes from the large intestine.* The small intestine does not produce mucus with the same facility as the large bowel. If mucus is found in diarrheal stools very finely subdivided, as in typhoid fever, tuberculosis, and cholera, it may be assumed that it comes from the small intestine. Further proof of the small intestine as the source of the mucus is the impregnation of the mucus with unchanged bilirubin.

The manner in which mucus is mixed with stools may indicate the source; if the well-formed stool is covered with mucus or if the mucus dips down between the scybala, it is coming from the lower end of the large intestine. The ribbonlike masses that are called membranous colitis mucus come from the sigmoid flexure of the colon. If mucus is intimately mixed with a ''soupy'' stool, the material comes from the upper part of the large intestine; in fact, the smaller the particles and the more intimately they are mixed, the higher up their source.

The pathologic process underlying the undue pouring out of mucus into the intestinal canal is usually a catarrhal process, with increase in the glandular activity and a diffuse inflammatory change in the entire mucosa. Mucous colitis is a mysterious disease entity which has been placed in the class of hypersecretion of the mucous glands with a nervous basis (a secretion neurosis).

The degree of inflammation may in a measure be determined from the transparency of the particles of the mucus. Our conception of a very severe attack is a mucus which is very cloudy, due to the content of epithelial and pus cells. There may, however, be a catarrhal inflammation without mucus in the stool, so that absence of mucus does not exclude inflammation. The appearance of the sagolike clumps of mucus occurs in the stools of children as an evidence of severe mucous inflammation. Heubner, in explaining this formation, thinks that the nodules of mucus are formed from the follicles in the intestinal wall, while Kelsch believes they are pieces that are squeezed out of the Lieberkuhn follicles. The presence of a large amount of pus with mucus indicates ulcerative inflammation; with blood, a diagnosis of ulcerative enteritis (dysentery).

Fibrin.—It is difficult to identify fibrin in the stools with the naked eye, or even chemically. Autopsy findings of fibrin are common in dysenteric patients, but there it is attached to the walls of the intestine. It is logical to assume that it is cast off and appears in the stool, but it cannot be proved by an examination of the stools. Fibrin masses are found especially in membranous colitis.

Tissue Remnants.—Tissue remnants emanate either from tumors of the intestinal wall or from necrotic mucous membrane changes. In the first group we note polypi, pieces of hemorrhoidal nodes, carcinoma, and occasionally lipoma. Necrotic pieces are seen in ulcerative processes; such as, invagination cases, typical dysentery, twisted off pieces of adenomatous polypi, or pieces of ulcerative neoplasms.

Very small necrotic masses have been seen in stools of typhoid fever in the second or third week. Occasionally we have received solid particles from a distance, which a physician thought was a piece of tissue. Usually these are artefacts or food remnants which passed out in undigested form. We have been asked to make sections of such pieces as well as sections of distinct blood clots. If we section the tissue and find actual glandular material we may well think of invagination.

The greatest stumbling block to an attainment of a good working knowledge of the material commonly seen in stools is the presence of nonpathologic substances or artefacts. These are fly larvae, unusual pieces of food, pieces of goods, pieces of apparatus, and other objects which may come in contact with the stools or containers. This must constantly be borne in mind; in fact, a microscopic study of all such foreign matter is very good preliminary practice in preparing oneself for coprological expertness.

Other Organic Material.—Other organic material may be parasites or various calculi. Among *calculi* are gallstones which are identified by their smooth and facetted surfaces and their light weight. Chemical means will fully identify them. While all sizes of gallstones may be found, the two extremes, very large and very small stones, are rare: the very large stones lead to obstruction and are not passed; the small stones are so easily broken up that they are no longer recognizable when they reach the lower end of the canal. The rare pancreatic stones have no known characteristics to make their identification with the naked eye easy. According to my old teacher Fleiner of Heidelberg, their surfaces are facetted and rough.

Enteroliths must be differentiated from so-called coproliths or fecal mass. Leichtenstern divided enteroliths into three classes: (1) heavy round stonelike masses with concentric layers when viewed in cross-section, showing centrally usually a foreign body as a nucleus; (2) light, irregular, porous stones, consisting of fecal and calcium salts with undigested vegetable food remnants; (3) concretions resulting from long-continued use of medicines, such as lime salts, benzoic acid, bismuth, salol. A curious habit of young girls of chewing the ends of their braided hair may lead to a fecal concretion with this hair as a nucleus. Very minute sandy particles of unknown origin have been reported in stools by the French authorities such as Dieulafoy.

The literature gives an occasional reference to the occurrence of *intestinal sand.* In 1903 Mitchell[1] reported a patient who passed sandy particles on a milk diet, the sand disappearing when the patient was put on a general diet. The sand was composed of calcium phosphate and carbonate. No bile or salts were present. Bedford[2] reports a case with severe mucocolitis coincident with the passage of sand. The analysis of the sand showed: moisture, 5.2 per cent; calcium phosphate, 28.68 per cent; calcium carbonate, 5.20 per cent; magnesium phosphate, 0.46 per cent; organic matter, 60.45 per cent. He states that the true intestinal sand, which is of mineral origin as opposed to false sand which is of animal origin, contains a higher percentage of inorganic salts than the false variety in which about 2 per cent is found. Delphine at the London Pathological Society in 1890 first called attention to the tendency of pears, figs, and bananas to produce false sand. He thought there was some relation between true sand and gout, but could not account for the absence of uric acid deposits. Crombie[1] reported several cases and stated that it is more commonly found in India because it is the custom there to wash all stools in the search for parasites. J. McNamara[2] looked at the condition from an evolutionary standpoint and suggested that the formation of sand is a reversion, in the mammal, to the eggshell forming function of birds and goes into comparative anatomy to prove his theory. J. S. Myer and J. E. Cook,[3] of St. Louis, reported a case of possibly false sand. They could not experimentally produce sand by the ingestion of vegetables. They produced it, however, by giving the patient one or two bananas, finding the sand twenty-four hours later in the stool. Fuller reported a case that he observed for a number of years, a married woman with diarrhea of many years' standing. She passed stringy mucus, and later passed two to three ounces of gritty sand. The sand was hard, crystalline, and yellow gray. It was partly soluble in nitric acid, turning to a bright orange color. It was insoluble in potassium hydroxide. The chief salts present were calcium phosphate and carbonate. It contained no bile salts. It could be washed from every stool. This writer concluded that most of the cases reviewed have been of a nervous disposition. He does not believe that diet has much to do with the condition, although false sand can be produced by large quantities of certain fruits. He suggested very properly that in all cases with persisting colicky pains the stools be washed and strained through gauze. This he believed will show a great many more cases than have previously been found.

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[1] Trans. College of Phys., Philadelphia, 1903.

[2] Indian Med. Gaz., 1902.

Microscopic Examination of the Stool

The microscopic examination of the stool may be made either a very simple procedure or a very complicated one. It depends upon how far we wish to go, and upon the object of our investigation. We may simply wish to survey the crystals present, or the food remnants, or to verify our naked eye judgment; or to prove the presence or absence of eggs of the more common parasites such as the hookworm or the tapeworm; we may wish to make a simple smear for tubercle bacilli, or to make a smear for other microorganisms; in short, our microscopic examination may be directed to the solution of one problem or a great many. We usually are given a clue from the clinician as to what he particularly wants investigated.

Preparation of the Specimen.—In general, the following material is needed for a microscopic examination of stools: glass slides and cover glasses; teasing needles; a good centrifuge; test tubes and small funnels, pieces of sterile gauze for filtration; sterile Petri dishes; culture media for identification of the more common varieties of bacteria such as the colon and typhoid bacilli.

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[1] London Lancet, 1902, p. 314.

[2] Brit. M. J., 1903.

[3] Am. J. M. Sc., 1909.

It is inadvisable to add any chemical substance to the stool to reduce the odor as these substances always cause a distortion of the picture and render the examination of doubtful value.

The simplest examination consists in taking a small piece of the stool on a toothpick and rubbing it on a glass slide, covering with cover glass and examining. Portions from various places should be taken, from the solid parts, and from softer and more liquid parts. Be sure the specimen is as fresh as possible to eliminate the question of alterations from standing. If the stool is not particularly fresh, take the specimen from the inside of the masses rather than from the outside. In exerting pressure on the cover glass elastic tissue and watery material will not remain pressed out, mucus and fat containing stools remain pressed out. If a grittiness as of glass against glass is noted crystals or sand may be present. Thin watery stools may be sedimented and thicker heavy stools may be slightly diluted with water or saline in making this examination. The mixture may be made on the slide. Examine with the low and high power dry lenses. To isolate certain parts of the stool, wash repeatedly, shake, sediment, and pour off the supernatant fluid. Remove the large particles which are visible to the naked eye and examine the sediment.

Sedimentation can best be accomplished with the use of a centrifuge tube very much like that devised by Hopkins for the counting of bacteria. Run the centrifuge at moderate speed. Note a sharp line of separation between the sedimented portion and the supernatant fluid. In the cloudy portion just above the sediment the various bacteria and the fatty acid needles are found in fat stools. Note foamy fat on the surface in cases with much fat in the stools. In this upper portion are seen small particles of vegetable material, mucus with air bubbles, and particles of connective tissue cells. In the deeper portions of the sediment are crystals of large size such as triple phosphates, the hairlike covering of vegetables, the large muscle remnants.

There are a number of other more elaborate methods of washing and sedimenting the stool for microscopic study but we feel that the above method will amply suffice for all practical purposes. We refer those who desire a much more elaborate method of separation of the various elements of the stool to Van Ledden-Hulsebosch's work.[1]

Microscopic Findings.—

Epithelia.—Epithelial cells from all portions of the tract are naturally likely to find their way into the stool. The character of the cell points to the origin, but pure histologic pictures as they occur in the mucosa do not exist in the stool because the digestive juices have a powerful effect in changing the morphology. There may be pure unchanged stratified squamous epithelial cells from the region of the anus but even this is rare. They appear, however, less changed than cells from points higher up; for instance, the cylindrical cells from the small and large intestines. These squamous cells closely resemble the cells that fall into saliva from the mucosa of the mouth. They are very large nucleated cells, and are not to be confounded with the nuclear-free cells of meconium and infant stools which have originated from the vernix caseosa. Pure types of squamous cells are rubbed off, unchanged, from the anal orifice by passage of the stool. Cylindrical cells from points higher up are seen as slightly distorted cells, found in washings of the gut or in thin soupy stools of typhoid, diarrheas of children, tuberculous enteritis, and cholera. They have clear nuclei and granular cytoplasm. They may appear singly or grouped as they are in the intestinal wall, arranged side by side. These cells are sometimes swollen with fat droplets. One may see the cells described by Nothnagel as ''vanishing'' or epithelial cell ''ghosts'' bound up with mucus particles. They are supposedly degenerated cells according to Leube, or amyloid cells according to Lambl. They are small, homogeneous, and have no nuclei. They are refractile, and apparently have lost their granules. They are frequently spindle shaped. Nothnagel believes they are cells which are changed by abstraction of water. Kitagawa, however, stated that he found these cells in watery stools in diarrhea and that they are the result of a degenerative process, either hyaline or coagulation necrosis. Schmidt[1] showed that they are caused by an inhibition with soaps, because, after adding acetic acid and gently warming the slide, fat droplets exude; further warming causes these drops to run together in a fatty film. Epithelial cells in the feces are usually not stained. Rarely are they yellow colored from bile. Nothnagel believed that the staining with bile is an intervital process and does not occur postmortem.

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[1] Makro u. microscopische Diagnostik der menschlichen Excremente, Berlin, J. Springer, 1899.

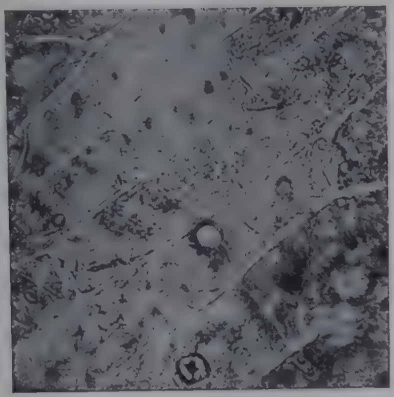

Fig. 313.—Vegetable cells in feces.

A sharpness in outline of the cytoplasm of the epithelial cells may be produced in the usual way by the addition of acetic acid. Osmic acid will stain fat granules black.

The diagnostic significance of epithelial cells in abundance is their indication of the existence of catarrhal processes. The occurrence of a large amount of mucus and a paucity of cells evidently shows that the derangement is more a result of a nervous irritability of the intestine than a true inflammatory process. This is especially true in cases of enteritis membranacea. Toxic disturbances of an intense nature, as in cholera, cause a great exfoliation of cells. One cannot conclude simply because the epithelial cells are bile stained that the disturbance is in the small intestine. Other signs must be at hand to make this diagnosis. The presence of the ''vanishing ghost'' cells does not prove their source the large intestine; when mucus is present as well, this is probable. The presence of half digested cells with mucus indicates a small intestine disturbance, especially if the cells are bile stained. This picture is seen in typhoid fever and tuberculous enteritis, where pus cells are also present.

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[1] Deutsch. Arch. f. klin. Med. 65: 2440, 1900.

Leukocytes.—Leukocytes are not commonly found in feces. They are abundant naturally where there is pus formation, as in ulcerative conditions in the tract. One may have difficulty in identifying them under the microscope but they may present a perfect contour, with granular cytoplasm and single or multiple nuclei. Eosinophilic granules may be seen; particularly is this true when Charcot-Leyden crystals are found in cases of parasitism. Leukocytes that are large enough to be designated giant cells have been seen in feces. They are much larger than the flat cells found in the mucosa of the mount. They show vacuoles. They are seen in tuberculous enteritis. Schmidt noted phagocytes in a case of dysentery. The shadows of leukocytes are sometimes seen. Small, broken down leukocytes are frequently seen enmeshed in mucous particles. The presence of a few leukocytes in conjunction with epithelial cells in infantile stools indicates an irritation process.

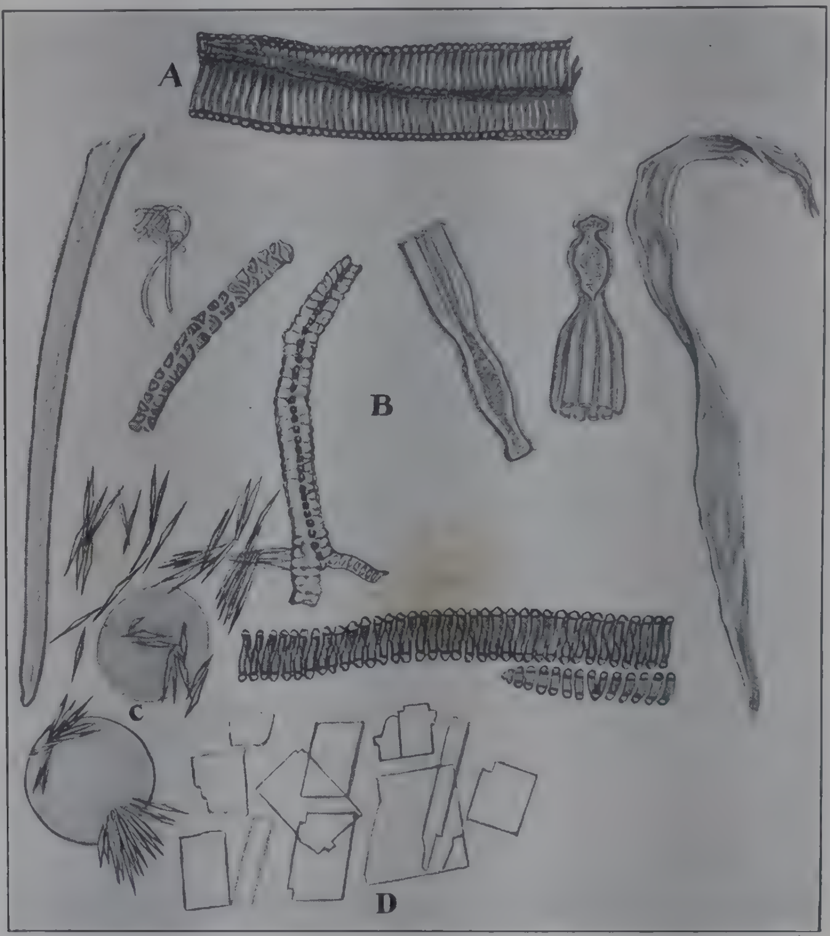

Fig. 314.—Microscopic findings in feces: *A* and *B*, vegetable fiber remnants; *A*, banana, commonly mistaken for tapeworm section; *C*, fatty acid crystals; *D*, cholesterol crystals.

Pus.—Macroscopically the grayish masses which microscopically prove to be collections of pus cells may be observed. If we actually see the yellow material which is unmistakably pus, we must think of para-intestinal pus pockets in the region of the large intestines. Pus coming from a point higher up in the small intestine would naturally undergo such changes before being expelled with the stool mass that it would not be readily recognized macroscopically. In the various ulcerative processes of the large intestine and even of the small intestine, such as dysentery, colitis ulcerosa, syphilis, neoplasms, tuberculosis, and typhoid, pus may be intimately mixed with the stool and is difficult to recognize as such.

Pus cells do not appear in feces in abundance in catarrhal states, as is the case in sputum of catarrhal inflammation of the respiratory tract. The presence of much pus always indicates an ulcerative pathology with but one exception; namely, colitis with severe inflammatory changes, with pus, blood, and mucus in the stools, without ulceration. One must remember that there are certain types of ulceration without pus; for instance, the perforation of typhoid ulcers. The various grades of proctitis with pus, blood, mucus, and Charcot-Leyden crystals have already been noted.

Erythrocytes.—Blood must be very fresh in order that the cells be seen in intact form under the microscope. Even then we usually see the crenated or crabapple form, due to the acid reaction of the stool. Only a large number such as are expected in an ulcerative or intensely inflammatory condition will yield shadow forms, which show no color. The finding of red cells in feces usually indicates ulceration. Exceptionally they are found in venous stagnation. Well-preserved corpuscles are seen in cases of hemorrhage from stasis low down (hemorrhoidal vein bleeding).

Detritus.—Besides the various elements already described, there are found in stools microscopic particles which are extremely small and impossible to identify. This material is called "detritus." It is intimately digested muscle remnants, plant cells, bacteria, epithelia, and isolated nuclei too far changed by the digestive juices to be recognizable. The presence of a large quantity of detritus is a good indication of the chemical and mechanical efficiency of gastrointestinal digestion.

Crystals.—Many crystals are found in feces, some derived from food, others from chemical changes in the intestine.

Triple phosphate or ammonium magnesium phosphate crystals are the commonest. They vary in size, some so small that they can be covered by calcium oxalate crystals, others very large. The best developed crystals are seen in fluid stools. Their shape is that of the "knife-rest" or "coffin-lid" but some are more or less irregular (see Figs. 33 and 34). Some are so large that they cover two microscopic fields, in which case they can be seen in the specimen with the naked eye, as a small white point on the slide. They sometimes appear as three- or four-cornered splinters representing breaking down of the "coffin-lid" formation. Triple phosphates are never bile stained. They are freely soluble in dilute acetic and other acids. They are insoluble in ammonium carbonate solution which distinguishes them from neutral phosphate crystals which they closely resemble. Some claim that they are found in normal and abnormal stools. Szydlowski found them absent in but one condition constantly; namely, icterus. Lynch states that they are never found in meconium. When found, we must be sure that there is no urine contamination.

Di-calcium phosphate or neutral phosphate of lime crystals appear in the form and size of those found in urine; namely, larger or smaller sheaves, with the points lying together. They may occur as rosettes. They are occasionally bile stained, whereas triple phosphates are never bile stained. A homogeneous transparent variety is seen in milk stools, this form being a combination with fatty acids. They are freely soluble in acids and in ammonia, as well as ammonium carbonate solution. They are found in normal stools, particularly when alkaline stools have been allowed to stand for some time. They have no diagnostic importance.

Neutral magnesium phosphate or trimagnesium phosphate crystals are found in stools showing ammoniacal decomposition. They occur as irregular sheaves, never as in urine as light refracting long rhombic plates. They are soluble in acetic acid, and become opaque when ammonium carbonate solution is added.

Calcium carbonate crystals appear as round or dumb-bell shaped crystals or amorphous granules. The addition of acetic or other mineral acids dissolves them with gas formation. Sulphuric acid causes them to assume a sheaflike form. These crystals are commonly seen in the stools of milk-fed children.

Calcium oxalate crystals are found in ingested vegetables that contain them; such as rhubarb, tomatoes, spinach, etc. They are not seen on a milk or meat diet. With a *large* amount of vegetables a moderate number have no significance; but with a *small* quantity of vegetables an enormous quantity of calcium oxalate crystals indicates an intestinal catarrh, showing that cellulose disintegration has been retarded, with an escape of the crystals from the plant cells. In hyperchlorhydria, an abundance of vegetables will not show many of these crystals, for these are dissolved by the excess of acid in the stomach; consequently there may be an increase of these crystals in the urine. They appear in various forms in feces, as envelope shapes, as typical rosettes, and as rhomboid and acicular varieties (see Fig. 35). They are insoluble in acetic acid and alkalies, but are soluble in mineral acids.

Crystals from Medicaments.—Dark bismuth crystals are found after ingestion of bismuth subnitrate. The use of salicylate of bismuth gives peculiar crystals of this drug; iron, charcoal, and manganese pass as black amorphous granules.

Cholesterin may at times appear as flat superimposed plates, often with re-entrant angles, but as they rarely show typical forms they must be identified chemically. They are easily soluble in hot alcohol, ether, and chloroform. They are insoluble in water, alkalies, and acids. On adding iodine followed by sulphuric acid there is a play of colors, yellow, yellow red, carmine red, violet, green, blue, with melting of the crystals from the edges. Cholesterin appears particularly in children where there is a large amount of mucus passed. It is commonly seen in meconium.

Charcot-Leyden Crystals appear as they do in sputum, as colorless octahedra with sharp margins and generally broken angles. They are commonly seen imbedded in mucus. Leichterstern spoke of them as especially characteristic of helminthiasis. His pupil, Buckler, further emphasized this relationship together with the presence of eosinophilia in the blood, but other authorities disputed this as constant or as sufficiently noteworthy to be of value in diagnosis. Charcot-Leyden crystals are found in abundance, it is true, in cases of helminthiasis, but they are absent in other cases. They have also been found in abundance in other conditions; to wit, in the mucus of enteritis membranacea, by Akerlund and Schmidt. They are no more characteristic of any disease when found in feces than when found in sputum, even though they are present in abundance in cases of bronchial asthma. Neubauer and Staubli[1] called the catarrhal inflammation of the large intestine with the presence of these crystals in large number an "eosinophilous proctitis."

Hematoidin and Hematin.—Hemin crystals are not seen in feces except after ingestion of blood sausage, but hematoidin crystals are common. They occur as reddish yellow rhombic plates, columns, needles, or bundles of needles, at times as amorphous masses. Uffelman reported them in infant feces and Lynch reported them in meconium. Von Jaksch found them in intestinal stagnation hemorrhages or free hemorrhages in adults. They are soluble in ammonia, the solution turning yellow. They are not soluble in potassium or sodium hydrate solutions. Nitric acid turns them blue.

Bilirubin appears like hematoidin as rhombic needle-shaped crystals or golden yellow kernels. They are found more frequently in infantile stools than in adults.

Uric Acid and Uric Acid Salts.—We doubt whether uric acid salts are ever found except as a result of contamination with urine.

Other Crystals.—Schilling[2] reported leucin and tyrosin crystals in feces in cases of pernicious anemia with chronic diarrhea.

Intestinal Parasites.—Parasites are living organisms which, for the purpose of procuring food, take up their residence in the living body. They may be of plant or animal origin; that is to say, phytoparasites or zoöparasites. We are here more especially interested in those parasites which take up their residence in the intestinal canal or structures adjacent thereto, and which may be detected by systematic stool examinations. Parasites might be occasional or permanent. We are here really concerned with the permanent parasites, for helminths, or intestinal worms, belong to this class.

Parasitism is relatively common in this country. It is much more common in tropical countries because of certain customs, failure to cook thoroughly, and also because many foods are ingested in a raw state. With the advent of rapid transport by air, many cases are directly imported into this country and are direct centers for infection of other persons. The spread of World War II into tropical lands resulted in many new cases among our military forces who were sent to these places for duty.

A full description of intestinal parasites may be found in Chapter XII.

Microchemical Reactions

Microchemical reactions are elicited by simply allowing a drop or two of a reagent to run in by capillary attraction between a cover glass and a slide after having made a smear of the feces. If the fecal mass is not fluid, it is impossible to do this, in which case drop the reagent directly on the preparation before placing the cover glass in position.

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[1] München. med. Wchnschr., 1906, No. 49.

[2] München. med. Wchnschr., 1900, No. 42.

The following *reagents* are necessary in this work:

0.6% Saline solution
Glycerin
Alcohol, ether, and chloroform
30% Acetic acid
3 to 5% Hydrochloric acid
Concentrated sulphuric acid
15% Potassium hydroxide
Millon's reagent (see page 48)
Modified Lugol solution

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	50 c.c.

1% Osmic acid
Sudan III (Herxheimer)
Equal parts of 70% alcohol and acetone saturated with Sudan III.
Sasthoff's Sudan III solution (glacial acetic acid 90 parts, 96% alcohol 10.0, a pinch of Sudan III).
Weak watery solutions of eosin, methylene blue, and safranin
Ziehl carbol-fuchsin
Concentrated watery solution of Nile Blue[1]

Polychrome solution of Friediger[2] made by mixing equal parts (2.0 c.c.) of concentrated alcoholic solution of dimethylamidoazobenzol, absolute alcohol, and 0.5 per cent alcoholic eosin solution, 20 drops mucicarmine, watery concentrated solution, 20 drops.

While it is not necessary to use all these reagents in all cases, one may adopt as a routine the following microchemical procedure in examination of stools.

Preparation of Specimens.—Make three preparations, one of the plain diluted feces, one mixed with acetic acid, and one treated with iodine solution. The first gives a good general picture. In the second, the striation of any muscle fibers is rendered more distinct; connective tissue is swollen up in contrast to elastic tissue which is unchanged; mucus is coagulated and rendered more distinct; cells are cleared and their nuclei appear sharper; albuminous granules are dissolved; some crystals, such as triple phosphate, disappear, while others, such as oxalates, are not affected. In the third preparation, undigested starch granules are colored deep blue; partially digested ones are brown or red.

Most of the material found in feces may be identified microscopically. However, there are certain very minute ingredients representing the breaking down of food remnants which we call "detritus" which cannot be identified.

Meat Fibers.—It is natural to find a certain amount of fibrous residue in the stool. Outside their mere presence, the quantity on certain diets is of great importance in drawing a conclusion as to the status of intestinal digestion. It is difficult to differentiate meat fibers from flesh, fowl, or fish; the coincident finding of scales of fish would suggest the fibers are of fish origin, while the presence of small pieces of feather would suggest fowl origin. The manner of cooking meat plays a part in the finding of meat fibers: well-roasted beef is more likely to remain in the stool than raw meat. Muscle remnants appear as isolated polygonal pieces devoid of sarcolemma. At times they lie together as if bound by the skeleton sarcolemma. They may be stained yellow from bile and thus attract one's attention immediately on looking through the microscope. Long and cross-cut striations are easily identified in the fibers. The size of these fibers varies from minute forms to pieces that entirely fill the field, under which latter circumstances they can be seen with the naked eye. The longer pieces have parallel sides, with cross striations.

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[1] Arch. f. Verdaumgskrankh. I. 1896, p. 396.

[2] München. med. Wchnschr. 1912, No. 52.

Better digested pieces do not show these sharp contours. The cross striations may be seen with the medium power dry lens. Schmidt states that cross striations in the fibers from fish disappear more rapidly than those from mammalian flesh. The color of fibers is dependent upon admixture of bile, the usual color being yellow or yellowish green. Nuclei can never be detected unless there is an absolute disappearance of pancreatic juice, in which case there is macroscopic evidence of unchanged meat in the stool.

Microchemical Tests.—It is difficult to identify meat fibers by microchemical methods because of the admixture of bile elements and the presence of certain unknown factors. One may attempt to remove bile elements by the use of alcohol; bilirubin-stained meat may be freed of this interfering factor with chloroform. Acetic acid swells the fibers and brings out the cross striations fairly well. The addition of potassium hydroxide causes the fibers to dissolve. If a little copper sulphate is added to the potassium hydroxide and the picture watched under the microscope, a slight violet color (the biuret reaction) may be observed. The addition of concentrated nitric acid with warming of the specimen gives a yellow color of xanthoprotein. Millon's reagent causes a disappearance of structure, with a red coloration. Picric acid and iodine-potassium iodide solution gives a yellow color. Eosin solution causes the appearance of a red color in meat fibers more quickly than in other parts of the stool.

There are are a number of very elaborate methods for exactly determining the amount of meat fibers present in any given stool, together with an estimation of the meat ingested after selected test meals. These methods are of doubtful usefulness. Certain conclusions may be drawn, however, from the presence of a large number of meat fibers in the stool. The process of digestion of meat occurs mainly in the small intestine, due to action of the pancreatic juices. No further digestion of meat occurs beyond the ileocecal valve. Hence the appearance of unchanged or slightly changed fibers in the stool is an indication not of large, but of small gut dysfunction. The disturbance in digestion in the small intestine may be motor, secretory, or resorptive in character. Unduly rapid peristalsis may account for large amounts of meat fibers in the stool, yet this very rapid peristalsis may be based upon a secretory disturbance in the pancreas and the intestinal glandular apparatus. In some cases, whether catarrhal, ulcerative or purely neurotic in origin, all three factors may be deranged. It is, therefore, extremely difficult to say which one of the functions of the intestine is materially deranged when too much meat fiber appears in stools. Summing up all the facts and reports in the literature on this interesting point, we may state that *the undue presence of meat fibers after a test meal indicates a fault in small intestine activity.*

Connective Tissue.—It is very easy to identify connective tissue in the stools with the naked eye, but it is still easier with the microscope. The connective tissue fibers are not found so constantly in feces as are muscle fibers. The finding of connective tissue is dependent upon the manner of cooking. They are found in the stools of infants on a milk diet; their undue presence indicates gastric disturbance. They are not found when cooked milk is ingested.

There are casein clots found under pathologic conditions in stools to which Nothnagel gave the name of ''yellow mucus'' bodies. They consist of casein clumps which resemble short pieces of muscle fibers; but they are not muscular in origin, for they are found three to four weeks after the patient ceases to take meat, as in typhoid convalescent states when no meat has been taken for weeks. They appear as minute yellow round bodies; under the cover glass they give an impression of sandy particles. Microscopically they appear as yellow particles or cells lying close together. There is no differentiation of structure with higher power. They contain no formed elements whatsoever.

They are dissolved by the addition of a mixture of warm water and potassium hydroxide. Acetic acid causes partial precipitation of the clump which is insoluble in an excess of this acid. The addition of potassium ferrocyanide solution causes a white precipitate; Millon's reagent stains them red. They are not found in normal stools. Their presence according to Nothnagel and others indicates a disturbance in small gut activity, usually an inflammatory condition.

Pieces of ingested egg may at times be seen in the stool as small particles of the skin or coagulated material (fried egg remnants). Meconium ''corpuscles,'' so called, are found under the high power magnification. They appear as weakly stained, greenish yellow, homogeneous, round or oval bodies lying near epithelia and cholesterin crystals. Schmidt believed they are shrunken epithelia of vernix caseosa.

Fat.—Fat substances are frequently found in the stool as neutral fat, free fatty acid, or soaps.

(a) *Neutral fat* appears in droplets or in scales of irregular form. The fat scales are usually unstained. The fat droplets appear round. Fat droplets are often stained yellow. In stools of infants fat appears just as it does in a microscopic picture of milk.

Microchemical Reactions.—All neutral fats when heated melt and run together; when cooled, they resume their individual shapes. Fats are insoluble in water, weakly soluble in cold alcohol, soluble in hot alcohol, ether, and chloroform. They are unchanged by the addition of potassium hydroxide. Osmic acid stains fat black, but not necessarily all neutral fats: olein and oleic acid are stained black; palmitin, stearin, and their corresponding acids are not stained except with the addition of alcohol. Fat in the animal body contains all these components. Therefore, osmic acid stains fat black, but with varying shades dependent upon the proportions of the ingredients just set forth. Substances other than fat may be stained black by osmic acid. The addition of a few drops of Sudan III stains fat red. The Friediger reagent stains fat yellow. Concentrated Nile blue reagent produces a rose red color.

(b) *Fatty Acids* appear as drops or scales and are difficult to distinguish from neutral fat, at times showing characteristic crystals. The crystals appear as fine needles with pointed ends, similar forms being seen in sputum. They also appear as lancet-shaped plates, occasionally seen in infantile stools or as needles which resemble soaps.

Microchemical Reactions.—They differ from neutral fats in that they are soluble in potassium hydroxide and cold alcohol. They are not bile stained in the stool nor do they take the stains. They are, however, colored by osmic acid and Sudan III like the neutral fats. They stain intensively with dilute carbol-fuchsin while neutral fats remain unstained. This is the best differential stain.

(c) *Soaps* appear as scales or crystals. They show less luster than neutral fats or fatty acids. They may be unstained or stained slightly yellow by hydrobilirubin or bilirubin. They usually appear as unstained needles. One variety is round with fine radial striations.

Microchemical Reactions.—The basis of these soaps is alkali. They are soluble in hot water and alcohol. They are mainly calcium salt combination. Upon heating the preparation they do not melt as do neutral fats and fatty acids. They are not stained by osmic acid or Sudan III.

Significance of Fat in Stools.—Infantile stools show a great deal of fat, as neutral fats, fatty acids, and soaps. Adult stools show fat, but usually only soaps as scales. They are not found in healthy persons after a test meal. Naked eye appearance of fat means an undue quantity. This fat can be seen as grayish, shining particles of a smeary consistency. Fatty acid crystals are never found in normal stools, but, according to Muller, are always seen in the stools of starving people. Fat as a pathologic entity is seen in stools in connection with disturbances of the biliary function, affections of the neighboring mucosa, or the mesenteric lymph nodes, where there is increased peristalsis without anatomic changes in the mucosa. Gastric and large intestine disease does not seem to have much influence on the undue appearance of fat in stools. Pancreatic disease does not necessarily show fat in the stool.

Fatty stools are sometimes seen in breast-fed infants, a mass of fat droplets with increased fatty acids and soap needles. Biedert called this fatty diarrhea. The addition of acetic acid and heat causes the entire microscopic picture to appear as fat droplets. The appearance of visible neutral fat in the stools of adults, of course, is pathologic except where castor oil has been taken.

Starch.—Unchanged or slightly changed starch granules with concentric or excentric markings seldom appear in the stools of adults. They are never stained by bile pigments; they are of varying size; and are usually changed by the process of digestion so that, except in stools of infants, one does not see starch that has not been subjected to chemical or physical agents. We must accustom ourselves therefore to the changed forms; chemical reactions usually are necessary to identify starch bodies absolutely. The microscope reveals bodies that are of different size from the original starch granule as it was ingested, granules that fail to show the classical markings. Even the ingestion of raw potato gives a starch residue that is not classical, with a corrosion or unevenness of the periphery.

Microchemical Reactions.—Cold water has no effect on starch granules, but warm water produces a slow swelling. Acetic acid has no effect. Iodine produces the well-known blue color on unchanged starch granules. We recommend the use of Lugol's solution for this test (described on page 1404). Rub the solution well into the fecal mass. The cellulose fiber present often hinders penetration of the iodine solution and prevents a reaction. Semidigested starch gives a mahogany red color indicating it has changed to erythrodextrin. In the final stage of digestion starch gives no color at all with iodine. Certain spores of fungi give a blue color with iodine. As stated above, unlike muscle fibers, starch is never stained by bile pigments.

As starch is present in some of the undigested vegetable remnants, care must be taken in the preparation of specimens to prevent these fibers from giving up their starch granules with consequent false interpretations. The presence of starch granules intact in the stools of adults points very definitely to derangement of the activity of the small intestine; hence one must be sure that the granules seen are eliminated as such and are not pressed out of vegetable residue. Starch in vegetable residue is found after the ingestion of peas and beans, especially in dried forms. Pancreatic insufficiency alone does not cause starch granules to appear in the stool. The activity of the intestinal bacteria is responsible for this fact; they evidently digest the starch when the pancreatic juices fail to do so. When, however, there is increased peristalsis with this insufficiency, starch is found in the stool mass. The appearance of starch granules, unchanged or only slightly swollen, therefore, indicates a much more severe grade of insufficiency of small intestinal digestion than does the appearance of muscle fibers.

After the ingestion of oily seeds such as almonds, in place of starch, or with it, are seen granular structures of a rounded or angular form known as ''aleurone'' grains. These are albuminoid in nature and give no blue color with iodine. They are freely soluble in water and pass out of the cell in which they occur in the intestine. As they are insoluble in serum they are seen frequently in stools of serous diarrhea and dysentery.

Cellulose and Vegetable Tissue

Recognition of plant cells and cellulose is important in examination of stools because they may be mistaken for animal remnants and parasites. There is some obscurity as to the exact changes which take place in the process of digestion of vegetable matter. Plant cells are covered or enclosed with cellulose which is commonly supposed to be quite resistant to the action of digestive juices. This is not altogether true, for Moeller has shown that thin cellulose walls are readily broken down in the digestive tract; the pectinlike material which forms the middle lamellae of plant cells is attacked in the intestine, the cells fall apart, and are thus exposed to further digestive activity. Cellulose found in stools is not chemically the same as when ingested, for, instead of the faint *blue* which they give with iodine and sulphuric acid before digestion, they show a faint *purple* coloration. They are also much more freely soluble in copper oxyammonia after digestion than they are before digestion. The nature of the change is probably a hydrolysis brought about by the action of bacteria, the alkaline contents of the intestine, and the intestinal ferments. The ferments from certain molds have the power of breaking down the toughest kind of cellulose; for instance, that of wood. The digestion of cellulose is aided by constipation because the bacteria have a longer period of contact under these circumstances, whereas in diarrheal states with rapid peristalsis, the cellulose is passed almost unchanged. Modifications of cellulose, such as the cutinized peripheral walls of stems and leaves and corky cells, are much more difficult to digest than unaltered material. For this reason they are seen more often in stools. Flat epithelial cells with the intervening stomata of leaves and also plant hairs are characteristic. The plant detritus most often seen is the lignified cells forming the spiral pitted and scariform vessels, and the vast fibers of the fibrovascular bundles. Young green vegetables give only spiral vessels, pitted and reticulated ducts appearing chiefly in old and over-ripe plants. Woody tissue, fruit envelopes of vegetables and fruits, pass through the intestinal canal practically unchanged and appear in stools as such.

Chemical Analysis of Feces

Fresh stools are usually used in chemical researches, but one may use dried stools satisfactorily. Chemical analysis is not commonly performed because of the time necessary for its completion and because not every case requires such an analysis. However, close study of gastroenterologic conditions have convinced us that an occasional chemical analysis gives information to the internist which cannot well be otherwise obtained.

Reaction.—Stools are ordinarily neutral in reaction although we have seen acid and slightly alkaline stools apparently under normal conditions. This acid reaction is due to the presence of lactic or butyric acid and usually is indicative of fermentation in the intestinal canal.

Litmus is the usual indicator. Moisten red and blue litmus paper with distilled water, place on a glass slide and rub on a fragment of feces, observing the change of color from the other side. Or, place a small amount of stool in a test tube, add sufficient water to make a suspension, shake well. Into another small test tube introduce a few cubic centimeters of a watery solution of tincture of litmus, add a few drops of the fecal suspension. Observe the color change. If the cloudiness of the fecal suspension interferes with the reading, centrifuge the suspension first and use only the supernatant fluid. A method published by Bruce[1] follows: Make a one per cent solution of alizarin. Place two small drops of the indicator on a glass slide about one and a half inches apart. Dip a stirring rod into the fluid part of the specimen, or, if the feces are formed, merely puncture the mass. Mix thoroughly in one of the drops using the other drop as a control. An alkaline reaction is indicated by a reddish violet to violet color, neutral no change, and acid, a light yellow. The density of the colors depends upon the amount of acid or alkali present. By placing the slide upon white porcelain, the depth of color may be more easily determined. After a few observations, it is easy to detect any abnormality in the reaction.

Changes in Reaction.—Meat diet gives an alkaline reaction, the more meat ingested, the stronger the alkalinity. Blood, mucus, and pus change the reaction. As putrefaction increases alkalinity and fermentative changes increase acidity, the reaction must be taken soon after passage of the stool. Excess of fat increases acidity from the presence of fatty acids. Catarrh of the upper intestine increases the acidity if peristalsis is rapid. Chronic processes of this kind, however, produce an alkaline state; for instance, in chronic enteritis, whether tuberculous or amebic. If there is a block in the pancreatic juice outflow, there are fatty stools with marked acidity. The presence of much protein may be masked by the ammonia formed, giving an alkaline stool. Block in bile outflow gives an acid stool, if there is associated pancreatic disease; if, however, there is no pancreatic disease, the stools are alkaline. Infectious hepatitis gives alkalinity.

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[1] Jour. Lab. & Clin. Med. 5: No. 1, 1919.

Reaction of infants' stools is dependent upon the diet. Breast-fed children have faintly acid stools. Bottle-fed children have neutral or slightly alkaline stools. The reaction is dependent upon the proportion of fat and protein present; i. e., when fat predominates the reaction is acid; when protein predominates it is alkaline. Therefore a breast-fed child with persistent alkaline stools shows that the mother's milk is possibly deficient in fats. The persistence of acidity in the stools indicates that there is an abnormal fermentative change.

"Dry" Constituents of Stools.—Normally the stool consists of watery parts, 65 to 85 per cent; dry solids 15 to 35 per cent. This is largely dependent upon diet, a meat diet increasing the solids and a vegetable diet increasing the watery parts. Under pathologic conditions the relative proportions of dry to watery constituents change; i. e., the dry part decreases when there is a block, the watery constituents increase, the most extreme condition occurring in cholera, where, according to the statistics of C. Schmidt, only 1 to 2 per cent of dry substances is found. Increase in watery constituents of stools is due to decreased resorption and increased secretions within the intestines. It might be termed a combination process, with the increase in transudation in the principal role. Schmidt believed that the extreme condition just noted, Asiatic cholera, is an example of blood transudation. An analogous condition obtains when senna or other purgatives are taken. The exact mechanics of these drugs is still in dispute, however.

Search for Bile and Biliary Constituents.—The pigment of feces which gives it its color is *hydrobilirubin* or *stercobilin,* formed from the bilirubin of the bile by reduction processes in the cecum and the upper part of the large intestine. This conversion of bilirubin into hydrobilirubin does not take place in man until bacteria appear in the intestine in early infantile life, usually at the time when the infant begins to take cow's milk.

Schmidt's Sublimate Test for Hydrobilirubin

Place a portion of fresh feces the size of walnut in a mortar and rub up with a small quantity of concentrated solution of bichloride of murcury (bichloride of mercury 25 grams, sodium chloride 2.5 gm., distilled water 500 c.c.). Allow to stand for twenty-four hours in a covered Petri dish. If hydrobilirubin is present, a deep red color develops in a few minutes. If only a trace is present it may take twenty-four hours to develop this color. This reaction may be obtained only if stools are quite fresh. Stools containing much fat must be extracted with ether before applying this test.

Schlesinger's Test

This is substantially the same as that for urobilirubin in urine, although some authorities deny the similarity chemically of hydrobilirubin and urobilirubin.

Shake a sample of feces with ether to remove the fat. Extract the residue with acid alcohol (hydrochloric acid 5 c.c., alcohol 95 c.c.); neutralize the yellow fluid that results with ammonia or sodium hydroxide solution. Mix with an equal quantity of 10 per cent zinc acetate in alcohol. Remove the precipitate, if any forms, by filtration through a dry filter paper. Examine the clear filtrate against a black background for the green fluorescence that indicates hydrobilirubin. Examine the red fluorescent solution with the spectroscope; it will show an absorption band between F and B which moves nearer to F when the fluid is acidified. No bands can be seen if feces contain blood.

These tests are used to prove whether stools are really acholic or not. A negative result with the zinc acetate test shows that no bile is reaching the intestine, and when associated with deep painless jaundice and progressive loss of weight and strength, is strongly suggestive of malignant disease of the head of the pancreas. Jaundice due to obstruction of the common duct permits a little bile to percolate through because there is a soft growth, whereas the hard growths at the head of the pancreas usually cause complete obstruction. Gallstones do not completely block the duct; infectious hepatitis does not completely block off the bile so that in these two conditions there is hydrobilirubin in the feces.

Bilirubin alone or mixed with biliverdin is encountered normally only in the meconium in the first week or two of life.

Gmelin's Test for Bilirubin

Add a few drops of nitric acid containing nitrous acid to a smear of feces on a piece of white porcelain; a play of colors, green, blue, violet, red, and yellow develops. If the stools are already green from the presence of chlorophyl or bacterial pigments, extract with water and test the aqueous extract.

Bilirubin is seen under pathologic conditions in diarrheal states, especially the jejunal diarrhea described by Nothnagel.

Bile acids, glycocholic and taurocholic, are completely reabsorbed from the intestine so that only a very small quantity appears in the lower part of the bowel. There, under the influence of putrefactive bacteria, they are split up into glycocoll, taurine, and cholalic acid. The cholalic acid combines to form cholate of soda which, under normal conditions, is the only representative of bile acids in the stool. If the intestinal contents pass so rapidly that the usual fission processes do not have time to take place within the large intestine, unaltered bile acids may be present.

Test for Biliary Acids

Extract a small quantity of feces with alcohol and filter. Distill the filtrate to drive off the alcohol, take up the residue with water rendered faintly alkaline with sodium hydroxide. Treat the watery solution with a trace of cane sugar, or a few drops of a dilute solution of furfurol, and add concentrated sulphuric acid. In the presence of bile acids a crimson color develops. The sulphuric acid must be added a drop at a time, shaking after each addition. Care must be taken that the temperature does not rise above 70° C., the test tube being cooled in water from time to time.

Watson's Test for Urobilinogen

This is fully discussed on pages 74 ff.

Spectroscopic and Chemical Tests for Occult Blood.—We have already discussed the presence of blood in the gross examination of stools, as well as its presence microscopically. Blood in extremely small amounts can be detected as "occult blood" by chemical or spectroscopic methods. These tests all depend upon the detection of one of the constituents of blood, hemoglobin.

The finding of occult blood in feces is of the greatest importance in the diagnosis of ulceration of the digestive canal. Hemorrhage from the nose, throat, esophagus, and hemorrhoids must be excluded. The finding of occult blood may establish whether an ulcer in the digestive tube is or is not healing. Bleeding from a cancer is *continuous* and bleeding from an ulcer is *intermittent.*

Significance of Occult Blood.—One must be guarded in the interpretation of a positive benzidine test for blood because it is difficult to differentiate exogenous from endogenous blood. The smallest amount of meat in the diet is likely to give a positive benzidine blood reaction. The patient must be kept on a meat-free diet for at least a week before making this test if the results are to mean anything. The physician who interprets this test must make certain that there has been no bleeding from the hemorrhoidal veins. The patient must be cautioned against brushing his teeth to the point of bleeding as the swallowing of even this small quantity of blood may cause a positive reaction in the stools. It is advisable, too, to restrict the amount of fat and chlorophyl-containing vegetables. A positive reaction means bleeding from any portion of the tract. In order to diagnose a case as ulcer of the stomach or pylorus, one must exclude bleeding from the nose, gums, mouth, throat, as well as the bleeding from portal or cardiac obstructions and from polyps and hemorrhoids as already stated. Blood is found in ulcus ventriculi and in malignant disease. During the suckling period blood is encountered in melena vera, hemorrhagic enteritis, from fissures, polyps, rectal papilloma, trauma, the hemorrhagic diathesis, invagination, and typhoid perforation. Duodenal ulcers are common in children and of course may lead to bleeding.

Spectroscopic Test

Snapper's method is the best.

Extract the feces in a mortar with acetone to remove the coloring matter. This leaves a dry mass.

Transfer the dry residue to a clean mortar, and rub up with a mixture of one part of glacial acetic acid and three parts of ether, preferably ethyl acetate.

Filter. Add one-quarter of a volume of pyridine, then a drop of ammonium sulphide solution.

Examine spectroscopically. If blood is present, a hemochromogen band will be found on the boundary between yellow and green.

Chemical Tests

The chemical tests are simpler. There are the guaiac, aloin, benzidine, and others.

Boas-Weber Test

Mix 1 gram of feces with 10 c.c. of 70% chloral hydrate in 95% alcohol.

Add 1 c.c. of glacial acetic acid and filter.

To 1 c.c. of the filtrate, add 1 c.c. of 2% guaiac resin in equal parts of chloral hydrate solution and hydrogen peroxide (3%).

A blue color indicates blood.

Kuttner and Guttmann Modification of the Weber Test

Stir 8 grams of feces with acetone. Let stand and pour off the supernatant fluid.

Treat the residue with 5 c.c. glacial acetic acid in 10 c.c. of 7% sodium chloride solution.

Extract with ether by adding the ether to the mixture, allowing it to settle, then decanting the ether.

Add a few drops of glacial acetic acid to the ether.

(1) Mix 1 c.c. of this with 2% guaiac resin,
(2) Mix 1 c.c. of this with 0.2% guaiac resin,
(3) Mix 1 c.c. of this with 0.02% guaiac resin.

All three are dissolved in equal parts of alcohol and oil of turpentine. The solution becomes **purple** if blood is present. The different concentrations are necessary because one cannot tell how much blood is present before making the tests.

Objection to the test: Less than 10% of blood in feces will not show a reaction.

Aloin Test

The aloin test is the same as above, except that a 2% alcoholic solution of aloin, to which turpentine has been added, is used instead of the guaiac solution.

A **red** color indicates a minimum of 5% of blood.

Pyramidon Test

Stir up a small part of the feces in 3 to 4 c.c. of distilled water. Filter. To the filtrate, add 3 c.c. of 90% solution of Pyramidon and several drops of acetic acid (30%) with hydrogen peroxide.

A **violet blue** is a blood reaction.

Boas Phenolphthalein Test

Reagent.—

Phenolphthalein	1 gram
Potassium hydroxide	25 grams
Distilled water	100 c.c.
Zinc powder	10 grams
Hydrogen peroxide, 3%	5 drops
Alcohol	2 c.c.

Technic.—

Stir 1 gram of feces in a mortar with 5 to 6 drops of glacial acetic acid and 10 c.c. of alcohol.

Filter.

Pour the filtrate on a mixture of 15 drops of Boas phenolphthalein reagent. A red color denotes blood.

Boas Thymolphthalein Test

Reagent.—

Thymolphthalein	10 grams
Potassium hydroxide	1 gram
Distilled water	25 grams
Zinc powder	100 c.c.

Technic.—Stir 1 gram of feces with 10 c.c. of alcohol and 10 drops of glacial acetic acid. Filter. To 30 drops of filtrate, add 30 drops of Boas thymolphthalein reagent and 15 drops of 3% hydrogen peroxide. A blue color denotes blood.

The Benzidine Test*

To make the benzidine reagent, dissolve 6 grams of benzidine base C.P. powder in 100 c.c. of 95% ethyl alcohol, and preferably allow to age 2 or 3 days before using. Collect fecal material in clean containers. Emulsify a small portion of the feces in distilled water, then pass through filter paper (Whatman No. 5 or more retentive paper) into a clean test tube to obtain a clear filtrate. If clouded due to breach in filter paper, refilter. To 3 c.c. of the filtrate add 8 drops of 50% aqueous acetic acid or glacial acetic acid, with mixing. Add 8 drops of hydrogen peroxide (C.P., 3%) to the resulting mixture and shake. Overlay the final mixture with an alcoholic benzidine solution, drop by drop, to obtain a contact ring. Tilt to bring about slight mixing at the ring. A positive reaction is indicated by the appearance of a greenish ring at the area of contact of the benzidine solution and the filtrate mixture, spreading into the benzidine solution either immediately or up to 2 minutes time, the intensity or gradient of color being in relation to the concentration of blood.

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*This method which is an improvement on other methods described is by M. B. Levin and J. Y. C. Watt: Rev. Gastroenterol. 16: 650-651, 1949.

When testing for blood in vomitus and gastric contents, add water in half the volume of material used, filter, and carry through the test as previously. In urine, when only a few red blood cells are found microscopically, centrifuge or sediment the specimen. Transfer the bottom 0.1 c.c., containing the sediment, to a clean test tube, add 0.4 c.c. of distilled water, and continue as previously described.

This method eliminates most of the substances producing false positive reactions as follows: (a) filtration of the stool suspension eliminates blood-containing particles of meats, oxydase-containing particles of foods, particles of animal charcoal, and insoluble metal salts (of iron, copper, etc.); and (b) the acetic acid destroys soluble oxydases. In the contact layer or ring test the soluble hemoglobin compounds give true positive blood reactions in dilutions up to 1:100,000; meat-free days are unnecessary preceding this test for occult blood.

The Benzidine Dihydrochloride Test.—This is described on page 84.

The Gregersen Benzidine Test

Use a powder consisting of 0.025 gram of benzidine and 0.20 gram of barium peroxide, kept in wax papers. Add the powder to a glass beaker and then add 5 c.c. of 50% solution of acetic acid. This liberates the hydrogen peroxide. As the powders keep indefinitely, and the solution does not, it must be made fresh for each test.

Procure a small portion of feces from the central part of the fecal mass to avoid contamination of blood from the anal region. If the feces have dried, moisten with water.

Spread on a glass slide; add 2 to 4 drops of the benzidine-barium-peroxide-acetic acid mixture. If pathologic blood is present, within one minute a **green** or **green blue** color appears. To be sure of the activity of the benzidine, Gregersen used a blood test paper as a preliminary measure. If when a drop of benzidine solution is placed on this paper, it turns deep blue, it is proof that the benzidine is in good condition.

Positive reactions are divided as follows: If it takes 15 to 60 seconds for a pale green or blue green color to show, it is a weak positive, or a 1 plus. Blood content is less than 1%.

If it takes from 3 to 15 seconds, with deeper color, it is a 2 plus. This is from 1 to 5%.

With an instant color development, it is marked 3 plus, and means 5%.

If benzidine dihyrochloride, 0.025 gram, is substituted for the benzidine base, a much more stable color is produced.

The patient is instructed to eat no meat, fish or certain vegetables, such as French fried potatoes, for at least four days prior to the test. Gregersen denied that chlorophyl-containing vegetables will give a false positive.

Detection of Blood by Formation of Hemin Crystals.—

Teichmann's Method.—Dissolve the suspected stain in a few drops of physiologic saline solution. If the suspected blood is in a liquid, evaporate slowly on a glass slide, and dissolve the residue in a few drops of salt solution.

Allow to dry. Place a cover glass over the dried stain. Run glacial acetic acid under the cover glass.

Heat *very gently* until bubbles begin to form, replacing the acid as it evaporates. Allow to cool slowly. After cooling, replace the acid with water.

Examine for hemin crystals with the high dry and low power objectives. The hemin crystals are dark brown rhombic plates, lying singly or in crosses. (See Fig. 292, page 1204.)

Lime, fine sand, iron rust interfere with the production of the crystals. Too much salt, too great heat, or too rapid cooling interferes with the formation of the crystals.

Protein Substances.—Coagulated albumin is not found in stools under normal conditions. Soluble albumin and albumoses are present only under pathologic conditions. Inasmuch as nucleoprotein is always present, being derived from the nuclei of the mucosa and food remnants, always remove it before testing for albumin.

Detection of Albumin and Albumoses (Schloessmann)

Grind up the feces with water and dilute to a thin fluid consistency. Allow to stand one hour and filter through a double filter. Mix the turbid filtrate with a little pure kieselguhr and filter. Acidify the clear filtrate with 30 per cent acetic acid, avoiding an excess. The precipitated nucleoprotein is removed by filtration once or twice through a double filter. If it filters clear, it is tested for nucleoprotein by adding a few drops of 3 to 5% acetic acid, but if it is turbid or gives reaction for nucleoproteins, add a little kieselguhr and repeat the filtration. The clear nucleoprotein-free fluid is now tested for albumin by adding cold nitric acid, by boiling with acetic acid after the addition of a little sodium chloride, or by the ferrocyanide or potash test. Simon suggested using small pebbles instead of kieselguhr because the latter takes up albumin.

Albumoses are tested for by the acetic acid and brine test. Make the specimen strongly acid with acetic acid and mix with one-sixth of its volume of concentrated sodium chloride solution. If much protein is present, a white precipitate appears, even when cold. If, on warming, the precipitate disappears, albumoses alone are present. If the solution contains both albumin and albumoses, the precipitate will disappear as the solution is further heated, and again show a turbidity when the boiling point is reached. On filtration of this hot fluid, the albumoses form a white cloud in the filtrate as it cools; the albumin will remain on the filter.

Biuret Test for Albumin

Mix the feces with water to a consistency of thin syrup. Test with litmus paper and add to each 10 c.c. a quantity of 10% acetic acid and alcohol, varying with the reaction: if the stool is strongly acid, add 0.15 c.c.; if faintly acid or neutral, 1 c.c.; if slightly alkaline, 1.5 c.c.; if strongly alkaline, 2.0 c.c. to 2.5 c.c. Mix well. Rub 5 c.c. of chloroform into the mixture. Place in a test tube and let stand until the chloroform and the larger particles sink to the bottom. Decant the upper yellow and faintly tinted fluid into another test tube and add a disk of copper sulphate agar. After one hour take out the disk, wash with water, and examine. If the feces contain a large amount of albumin, the disk retains its bright blue color, but if no albumin or only a trace is present, it will be brownish blue. On pouring over it a little diluted solution of potassium hydroxide, the color will change to blue violet if albumin is present.

Copper sulphate agar is made by boiling 2 grams of agar in 100 c.c. distilled water until dissolved, then adding 10 c.c. of a 10% solution of copper sulphate. Pour the hot mixture into a glass tube 0.8 to 1.0 cm. in diameter, 20 to 30 cm. long, closed at one end by a plunger. Close the other end with a rubber cap. To make a test, remove cap, push in the plunger, and cut off a disk about 1 cm. long.

Caution: Hydrobilirubin also gives the reaction. Ury noted that other pigments and the remnants of paranuclein and casein give misleading results.

A simple test will be found on page 62.

Dissolved albumin is found pathologically only with inflammatory conditions. Albumoses are found only under rare conditions and always in association with albumin. They are found more frequently in stools of babies, but here they undoubtedly arise from the food ingested and not from the intestinal wall.

Casein.—No satisfactory test for casein has been devised. It is better to rely upon macroscopic tests than upon chemical analyses.

Nucleins.—Nucleoproteins are combinations of nucleic acid with a protein substance. Nucleins may be broken up into purine bases such as guanine, adenine, xanthine, hypoxanthine, and uric acid. They may arise from remnants of nuclei of food, secretions of the mucosa, juices poured from the alimentary tract by the glands opening into it, and bacteria of the intestinal canal.

Nucleins and purine are tested for as follows: Dilute the entire day's feces with 1 to 2 liters of water acidified with 15 to 20 c.c. of concentrated sulphuric acid, and heat for three hours over a free flame in a flask with a reflux condenser. Make alkaline with sodium hydroxide, acidify with 10 c.c. acetic acid, and heat for a short time on the water-bath. While hot, add 10 grams oxalic acid and an excess of chalk. Cool, make up to 1,500 c.c. with water, and filter. Wash off the residue with hot water, mix with acid solution of sodium acetate, warm, filter, and add the filtrate to the previous filtrate. Place 500 c.c. of filtrate in a flask, make alkaline with sodium hydroxide, mix with 10 c.c. of a solution of sodium bisulphite for each 100 c.c. of fluid, and boil. Add an equal quantity of 10% copper sulphate; boil for a few minutes. Filter, wash the residue on the filter into a flask, make up to 200 c.c. with water and shake well. Boil and add sodium sulphide solution previously treated with 1% sodium hydroxide to free it from sulphuretted hydrogen, until lead acetate paper no longer gives a brown color. Boil, add 10 c.c. acetic acid, heat until the copper sulphide is precipitated. If this does not occur, heat with 5 to 10 c.c. of saturated solution of aluminum acetate. Filter, mix residue with water, boil, filter, and add filtrate to that first obtained. Repeat several times. To the mixed filtrate add 10 c.c. of a 10% solution of hydrochloric acid, evaporate to dryness. Treat residue with 5 c.c. hydrochloric acid, and a little warm water; heat, cool, and filter. Wash the flocculent precipitate with water, test for purine bases with the copper or silver methods (for this method see section on test for uric acid under blood chemical methods).

Mucin.—Mucin is a compound protein radical and a nitrogen-containing carbohydrate, glucosamin. Boiling with acids makes mucin yield a copper-reducing substance like a sugar. Mucin dissolves in weak alkaline solutions and is precipitated by acetic acid. It may be identified by acetic acid, with microscopic study. The mucin becomes cloudy and develops lines. This is a better method than the chemical methods.

Protein Derivatives.—

Amino Acids.—The digestion and putrefaction of protein substances in the intestine yields a number of very interesting amino acids. These are leucine (amino-isobutyl acetic acid), tyrosine (oxyphenyl-amino-proprionic acid), and tryptophan (indol-amino-propionic acid). These undergo further changes, breaking down into phenol, paracresol, indol, and skatol, which appear partly in urine with sulphuric and glycuronic acids, and partly in feces in an unchanged state. Lack of pancreatic action causes leucin and tyrosin to appear in feces.

Gelatin Film Test for Stool Trypsin.—

The gelatin film test estimates roughly the proteolytic activity of stools by determining their ability to liquefy gelatin. Fecal suspensions are placed upon gelatin film (unexposed and unfixed) and the extent of removal of the gelatin film is noted.

In determining the significance of the test, Schwachman, Patterson, and Laguna[1] studied two groups of patients: (1) 500 normal and sick individuals; and (2) 50 patients with pancreatic fibrosis. In group 1, less than 5 per cent of the infant group lacked this proteolytic power. As one advanced into the older age groups, this percentage increased. The administration of a laxative for some older children without tryptic activity resulted in stools possessing tryptic activity.

A total of 220 separate stool specimen was examined from patients in group 2. In 209 specimens, there was no tryptic activity. The remaining 11 specimens from 7 patients showed definite tryptic activity. In this group, 3 patients had minimal tryptic activity in the duodenal fluid.

When oral pancreatin was given to patients with pancreatic fibrosis, the stools became positive for trypsin. When laxatives were given to a few patients with pancreatic fibrosis, the stool trypsin remained negative.

Conclusions and interpretations based on a single examination are hazardous. At least three specimens should be studied to eliminate or to make probable the diagnosis of pancreatic insufficiency, and whenever doubt arises, duodenal intubation is indicated.

There are two procedures, a test tube method, and a gelatin film method. The test tube method is more reliable and reproducible due to the fact that the film substrate in the gelatin film method might change its properties when exposed to different temperatures and if stored for varying periods of time. However, the film method offers the advantage of simplicity and convenience, and can be used as an office test. Photofilm is less reliable than gelatin film.

(1) Test Tube Method.—

Reagents.—

5% Sodium Bicarbonate Solution.—

Dissolve 5 gm. sodium bicarbonate ($NaHCO_3$) in distilled water and dilute to 100 c.c. with distilled water.

7.5% Gelatin Solution.—

Dissolve 7.5 gm. of gelatin in 100 c.c. distilled water heated to about 37° C.

Technic.—

Place 4 c.c. of distilled water in a test tube graduated at 5 c.c.

Add feces to the 5 c.c. mark.

In another test tube graduated at 10 c.c., place 9 c.c. of distilled water.

Add feces to the 10 c.c. mark.

This gives two samples of fecal material diluted, respectively, 1:5 and 1:10 with distilled water. Further dilutions can be made as desired.

Mix the contents of the two tubes thoroughly.

Place 1 c.c. of the 1:5 dilution in one tube and 1 c.c. of the 1:10 dilution of feces in another tube.

Add 1 c.c. of 5% sodium bicarbonate,

and 2 c.c. of 7.5% gelatin solution, previously warmed to 37° C., to each of the two tubes.

Mix well and place the tubes in a 37° C. water bath for 1 hour.

Then transfer both to an ice bath for 30 minutes.

At the same time, prepare a control tube containing all the ingredients except the feces and carry it through the same procedure.

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[1] Schwachman, H., Patterson, P. R., and Laguna, J.: Pediatrics 4: 222, 1949.

Examine all tubes at the end of the 30-minute period of ice bath incubation.

If the contents remain liquid, record the tryptic activity as 4+.

A rigid gel indicates no activity.

Compare the tests with the control.

(2) Gelatin Film Method.—

Prepare stool dilutions in water as described in method 1.

Place 1 large drop (approximately 0.1 c.c.) of the well-mixed specimen on an unexposed, unfixed gelatin film such as is used in routine hospital practice. The film strip may be clipped to a cardboard to prevent curling.

Incubate the film at 37° C. for 1 hour. Although 37° C. is the recommended temperature, room temperature is satisfactory if the incubation period is prolonged to 1½ to 2 hours.

Wash the film in a stream of cold water with gentle rubbing.

The result is now apparent. A clearing at the site of the drop indicates enzyme activity. A complete clearing is designated as 4+, and a slight clearing only at the periphery of the drop as 1+.

If the drop is small or if the concentration of the specimen is too great, the material will dry and cake.

Proper bacteriologic precautions should be taken when dealing with cases suspected of enteric infections.

Both a 1:5 and a 1:10 dilution of stool are used routinely.

When performing the test for the first time, or when using new gelatin film, adequate controls should be run. Determination of the end point using normal infant stools or a standard trypsin solution is suggested.

Discrepancies between the film and tube methods occur in less than 1% of the tests. The tube method is more sensitive. For example, a specimen showing a 2+ reaction in the 1:10 dilution and a 4+ reaction in the 1:5 dilution on the film will be strongly positive by the test tube procedure using a 1:10 dilution.

Test for Tryptophan

First extract the feces with ether, then with alcohol; filter; evaporate to dryness; dissolve the residue in hot water. The amino acids crystallize out. They appear in characteristic form.

Fischer and Bergell's Test for Amino Acids

Rub up feces with water, then with acetic acid; boil and filter. Concentrate on the water-bath, cool, and shake eight hours with ether in a shaking machine to remove the fat. Decant the ethereal portion and mix the residue with potassium hydroxide, add 25% sulphuric acid and 20% phosphotungstic acid. Filter. Add barium hydroxide to the filtrate, concentrate it on the water-bath, and for every 500 c.c. of the original fluid add 2 c.c. of a 10 per cent solution of naphthalene-sulpho-chloride. Make slightly alkaline and shake for twelve hours in the shaking machine. Every three hours 1 c.c. of the reagent and a little potassium hydroxide are added. Flood the product at the end of the shaking with ether, acidify the supernatant fluid, and allow to stand 24 hours. At the end of that time a crystalline deposit of naphthalene-sulpho-tyrosin is formed. It is readily crystallizable from alcohol, melting at 129° C. The watery portion gives a strong Millon's reaction.

Phenol, Indol, Skatol.—These and other aromatic oxy-acids appear in stools as a result of putrefaction.

Rub feces to a thin mass with water and distill. This distillate contains these products and fatty acids. Neutralize with sodium bicarbonate and distill again. The fatty acids remain behind. To separate the phenol from the others, make distillate alkaline with potassium hydroxide and redistill. This distillate contains the indol and skatol. To obtain the phenol in the residue, make it acid with sulphuric acid and redistill.

Aromatic oxy-acids are detected by taking the residue from the first distillate, adding sulphuric acid, extracting with several portions of ether, evaporating off the ether, dissolving the residue in a little water, and testing with Millon's reagent. A red color on warming results.

Phenol is identified in the distillate by a red color or precipitate with Millon's; amethyst blue on the addition of a few drops of dilute neutral solution of perchloride of iron; and the formation of a turbidity, followed by a precipitate of yellow white needles or a cheesy mass of tribromphenol when bromine water is added.

Indol is tested for in the distillate by treating it with a few drops of nitric acid containing nitrous acid, when a red color and later a precipitate forms, due to the formation of nitroso-indol. On adding a few drops of sodium nitroprusside, and a little sodium hydroxide, a violet color appears which turns blue upon the addition of glacial acetic acid. A pine wood splinter moistened with strong hydrochloric acid turns bright red with an alcoholic solution of indol.

With **skatol** in the distillate, nitric acid containing nitrous acid causes a milky cloudiness to develop; on adding sodium nitroprusside, a yellow color develops, which, on boiling and adding one-half of its bulk of glacial acetic acid, turns violet. Skatol dissolves in hydrochloric acid with the formation of a violet color.

Evidence of intestinal putrefaction is usually sought for in urine rather than in feces; i. e., the test for indican in urine is most commonly used. The evidence of intestinal putrefaction from stool examination must be very carefully interpreted. These products are formed from protein matter, principally from meats. Rapid peristalsis prevents their occurrence. They are present in the largest amounts in constipated individuals on a meat or protein diet. The presence of hydrochloric acid in the stomach has an important bearing on putrefaction. Putrefaction can be increased by lowering the acidity by administering alkalies or atropine, and by giving a diet rich in albumin. As already stated, possible better and quicker results can be obtained by testing for indicanuria.

Cadaverine and Putrescin.—Cadaverine is pentamethylenediamine. Putrescin is tetramethylenediamine. They were first found in feces in 1888 by Udranszky and Baumann.[1] These two substances belong to the class of ptomaines. They were found originally in a case of cystinuria. They are formed from putrefaction of proteins and are related to the diamino acid, lysine, and ornithin. The presence of these two substances is naturally dependent upon protein decomposition. Some maintain they are formed as a result of bacterial activity within the intestinal canal. The tests are intricate and the clinical deductions mean but little. We will, therefore, not devote any space to the test, except to refer to Udransky and Baumann's original article (l.c.). While on this subject of the toxicity of fecal matter, we might refer to the work of Magnus-Alsleben[2] and Rogers and Carneier[3] that the injection of fecal matter from dogs and rabbits intravenously showed strongly toxic characteristics; also to the work of le Play[4] that the same results follow upon the injection of infantile stools.

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[1] Ztschr. f. phys. Chem. 13: 562, 1889.

[2] Biochem Ztschr. 6: 503, 1905.

[3] Compt. rend. soc. Biol. 2: 388 and 674, 1905.

[4] Les lesions intestinaux. Thésé de Paris, 1906.

Fat.—In a consideration of fat in stools we refer actually to the higher insoluble fatty acids. To the casual reader the fat in stools is closely related to the fat ingested and the ability or lack of ability of the normal physiologic processes to handle it. This is true in the main, yet, according to Williams' researches, there may be a general metabolic disorder which throws on the intestine the onus of eliminating some of the fatty acids, calcium salts, etc.; i. e., some of this fat is endogenous and not exogenous. As a rule, we look to the naked eye appearances and to the microscopic examination for a general outline of fat content. The most important conclusion to be drawn from the presence of a large amount of fat in stools is a block in the outflow of bile into the intestinal canal.

According to Biebert, the presence of more than 40 per cent of fat in stools indicates a condition of fat diarrhea. In normal dried stools of infants the fat should not exceed 20 per cent. Increase in fat content to a marked extent is always a biliary or pancreatic affair. Pancreatic juice has for its principal function the digestion of fats, which is markedly increased by the presence of bile and still more by the presence of both bile and hydrochloric acid. Bile of itself can do nothing with fat, but a mixture of bile and pancreatic juice can digest three times as much fat as pancreatic juice alone could do. Wohlgemuth states that the lipase or ferment of the pancreatic fluid exists in an inactive form and that it is activated by the bile. The bile also helps to dissolve the free fatty acids formed by the action of the pancreatic juices lipase on neutral fats, for these acids are insoluble in water but very soluble in bile: bile therefore assists in the absorption of fats.

No data of any value can be obtained from a chemical analysis for fat unless dried feces are used.

Place a required quantity of feces in a shallow porcelain dish of known weight, and carefully weigh. Transfer the dish and contents to a water-bath kept at 50° to 60° C. After most of the water has been driven off, break up the residue with a glass rod. Return the dish to the water-bath and heat for another half hour. Remove the feces from the dish, transfer to a porcelain or glass mortar, grind to a fine powder. Return the powder to the dish and heat for another half hour. Cool the dish and weigh, repeating the process. Two consecutive weighings must give the same results. This figure gives the weight of the dried feces from which to deduce the *water content*. If the stool contains much fat, it is difficult to dry it because the fat forms a film on the surface which interferes with evaporation. To avoid this, lower the boiling point by the addition of alcohol from time to time. Heat on the water-bath for three to four hours in a porcelain casserole. Add 50 c.c. of absolute alcohol, stir with a glass rod and again heat. In one hour add 25 c.c. more alcohol, stirring well, and evaporate as before. Add a third portion of alcohol, stir, and evaporate for another hour. Heat on the water-bath at 108° C. for a short time, thus removing all the water. When a constant weight is obtained there is no more water present.

Another rapid method of drying for fat estimation only: Mix the stool with 10 volumes of silver sand (Merck's purified sea sand) which has been successively washed in hydrochloric acid, water and alcohol and dried. Dry at 100° C.

Test for Total Fat

Neutral fats and free fatty acids are soluble in ether; combined fatty acids or soaps are not. To include both in the analysis, one must break down the latter and set free the fatty acids with a mineral acid. Place 0.5 gram of dry powdered feces in the lower bulb of the Schmidt-Werner milk tube with the use of a funnel. Pour 10 c.c. of diluted 30% hydrochloric acid into the tube to carry down the particles that may adhere to the sides. Heat in a boiling water-bath for 15 minutes, rotating occasionally to mix contents. Cool, fill to the 50 c.c. mark with ether, securely cork, invert forty or fifty times, rotate between hands to get rid of air bubbles, fix in upright position, allow to stand a half hour to give time for all the solid particles to sink to the bottom of the tube. Rotation of the tube occasionally hastens this process. Pipette off 20 c.c. of the clear supernatant fluid and place in small weighed beaker. Evaporate the ether, dry the residue on a water-bath, cool, and weigh. The difference in weights is the weight of the fat contained in 20 c.c. of the ether. Read off the height of the liquid in the tube after the 20 c.c. have been withdrawn.

Calculate the percentage of fat as follows:

If $w =$ the weight of the feces taken
$x =$ the weight of fat found in 20 c.c. of ether
$y =$ the number of c.c. left in the tube

then $(y - 10)$ will be the amount of ether left in the tube, for 10 c.c. were dilute hydrochloric acid, and $20 + (y - 10) = (y + 10)$ will be the total bulk of the ether in which the fat from "w" grams of feces was dissolved.

$$\frac{x\ (y + 10) \times 100}{20 \times w} = \text{the percentage of fat in the stool.}$$

Besides the total fat, the ether extract of the feces contains the lower fatty acids, cholesterin, lecithin, cholalic acid, pigments, etc. These exist in quantities so small that they are negligible.

Tests for Neutral Fat, Free Fatty Acids, and Soaps

Neutral Fat and Free Fatty Acids are soluble in ether, whereas the combined fatty acids or soaps are not, and must be broken down by the addition of a mineral acid to make them soluble.

Test for **neutral fats** in the following manner: Place 0.5 gm. of dried feces in the lower bulb of the Schmidt-Werner tube; add 10 c.c. of distilled water; heat the tube for fifteen minutes in a boiling water-bath, rotating the tube occasionally to get a good mixture. Cool; fill to the 50 c.c. mark with ether; carry on the remainder of the test of the feces for the total fat and the fatty acids and neutral fats side by side, labeling the tube "A" which has been treated with acid,* and the tube "B" which has been treated with water. The ether extract of tube B gives the sum of the neutral fats and the fatty acids (free). The contents of tube B gives the unsaponified or, as Cammidge speaks of it, the "unsoaped fats," while the difference between the two gives the combined fatty acids or soaps. To determine the relations between the neutral fats and free fatty acids, dissolve the ether extract from tube B in an excess of ether (about 20 c.c.), add a few drops of an alcoholic solution of phenolphthalein; then titrate with a decinormal sodium hydroxide in alcohol. The number of cubic centimeters used to neutralize the solution, multiplied by 0.0284, gives the weight of the free fatty acids contained in the quantity of feces originally used. The difference between this and the weight of the ether extract gives the quantity of neutral fat (and lipoids).

There are a number of other tests for fat which are sufficiently important to be carried out in certain cases.

The **acid test** is the number of milligrams of potassium hydroxide necessary to neutralize a gram of fat; this is a measure of the degree to which the fat has been hydrolyzed. Weigh a small amount of the fat, dissolve in neutral alcohol or alcohol-ether; add a few drops of 1% alcoholic phenolphthalein, or if the fat is dark in color, a 2% solution of alkali blue 6 B; titrate with tenth or half normal aqueous potassium hydroxide.

The **saponification value** is obtained by determining the number of milligrams of potassium hydroxide necessary to neutralize the fatty acids split off from one gram of fat on saponification. Boil a weighed quantity of fat with alcoholic potassium hydroxide on a waterbath for half an hour. Titrate the alkali in the flask with half normal hydrochloric acid, using phenolphthalein as an indicator. Compare the result with that given by a control experiment in which the alcoholic potassium hydroxide only was used. The difference gives the amount of alkali neutralized by the fatty acids liberated from the weighed quantity of fat, and from this the saponification value can be calculated. As the soaps formed are hydrolyzed by water, and, therefore, react alkaline, it is necessary to add neutral alcohol from time to time in sufficient amount to keep it at over 40% since hydrolysis of soaps does not occur in this strength of alcohol. The saponification value is a measure of the mean molecular weight of the fatty acids entering into the composition of the fats. By means of it the nature of the fat present is suggested. Fats such as tallow or lard give a saponification value of about 195, butter 220 to 233, cocoanut oil 250, and cod liver oil about 171 to 189.

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*Test for total fat.

The **ester value** is the difference between the saponification and the acid values of the fat, and is therefore the number of milligrams of potassium hydroxide necessary to neutralize the fatty acids present in the form of neutral esters.

The **iodine value** of a fat is the amount of halogen, reckoned as iodine, that the unsaturated fatty acids of this fat will absorb, expressed as a percentage of the weight of the fat. The iodine value of a fat or a mixture of fats is, therefore, an index of the proportion of unsaturated or saturated fats present, for the latter do not absorb iodine. In feces, oleic acid, with an iodine value of 90.1, is the most important unsaturated fatty acid. Feces of children fed on cow's milk have a lower iodine value and a higher melting point than of those fed on mother's milk. The first stools of the latter after the meconium has been voided are particularly rich in this substance. They have an iodine value of 48% and a melting point of 40° C. At the end of the first week the proportion of oleic acid sinks, and the melting point and iodine value approximate those of older children; namely, 47° C. for the former and 20% for the latter. In cases of dyspepsia, the iodine value rises and the melting point sinks.

Method: A titrated solution of iodine monochloride, obtained by mixing solutions of iodine trichloride and iodine in glacial acetic acid, is added to a solution of the fat in carbon tetrachloride. This, after standing in the dark for one to two hours, is mixed with a 10% solution of potassium iodide, and is then titrated with a standard solution of sodium thiosulphate. From the amount of iodine absorbed by the known weight of fat taken the iodine value can then be calculated.

Hehner's value is the percentage of fatty acids insoluble in water given on saponification of the fat. For triolein, the Hehner value is 95.7, for lard and most fats and oils 95, for butter between 86 and 88, and for cocoanut oil, still lower.

Saponify a weighed quantity of fat with alcoholic potassium hydroxide, evaporate the alcohol, dissolve the soap in hot water, and make the solution acid with hydrochloric acid. Heat until the aqueous layer is clear, and filter. Wash the fatty acid with boiling water as long as any acid reaction can be detected, then dry and weigh.

The **Reichert-Meissel value** is the number of cubic centimeters of decinormal potassium hydroxide necessary to neutralize the volatile acids produced by the saponification of 5 grams of the fat, and is a measure of the amount of lower fatty acids (which volatilize in a current of steam) that enter into the composition of the fat. To obtain comparative values, the experiment must be carried out under certain prescribed conditions, which vary somewhat in different countries.

The **acetyl value** gives the number of milligrams of potassium hydroxide necessary to neutralize the acetic acid liberated when one gram of acetylated fat or fatty acids, formed by heating the fat with twice its weight of acetic anhydride, is saponified. Animal fats do not give an acetyl value unless they are mixed with cholesterol or some alcohol soluble in water, but certain vegetable oils have high acetyl values; thus, for castor oil, it lies between 153 and 156.

Total Nitrogen.—

Use the Kjeldahl method. The ammonia nitrogen and protein nitrogen are converted into ammonium sulphate by heating with sulphuric acid. The ammonia is set free with an alkali and the distillate is titrated with a standard acid. To destroy the organic matter, mercury is added to the sulphuric acid mixture. Use dry stool or about twice the weight of fresh stool.

Weigh two grams of dried feces and place in the bottom of a Kjeldahl flask.

Add 800 c.c. of pure sulphuric acid, 200 c.c. of fuming sulphuric acid, and 100 grams of anhydrous phosphoric acid free from nitrogen. Add about 0.1 c.c. mercury from a pipette; place flask aside for twelve hours after shaking it, as it will foam too much if heated at once. Heat in the usual inclined position on a sand bath or asbestos mat with a small flame, later with a full flame, until the fluid is clear. This takes three to four hours. Shake down any particle that comes up on the neck of the flask. Cool and wash the contents into a 500 c.c. distillation flask with 50 c.c. water. Wash the residue into this flask with fresh portions of water, keeping the total contents below 200 c.c. Cool the flask, add 50 c.c. of 10% sodium hydroxide solution, 40 c.c. of 4% potassium sulphate solution, and a spoonful of talc or a few zinc filings. Add 60 c.c. of the sodium hydroxide solution, connect quickly with the condenser. The delivery end of the condenser dips into a small flask containing 50 c.c. of fifth normal sulphuric acid. Heat and distill. Disconnect and stop the heating in 20 minutes. Wash the delivery tube with a little distilled water into the receiving flask, adding a litle cochineal or methyl orange solution. Titrate with N/5 sodium hydroxide. The number of milligrams of nitrogen in the dried stool is obtained by multiplying the number of c.c. of N/5 sulphuric acid bound by the distilled ammonia by 2.8.

The interpretation of the amount of total nitrogen found is difficult: The nitrogen comes from the proteins of food, from the secretions and metabolism of the gastrointestinal tract, and from bacteria. Schmidt and Strassburger consider that on a diet yielding only a small amount of residue, bacteria furnish no less than a quarter, or even half, of the total nitrogen of stools. In general very little help is obtained in the diagnosis of pathologic conditions from total nitrogen determinations.

Carbohydrates.—

Sugars do not ordinarily appear in stools of adults. Interference with absorption may cause them to appear in stools of children. Sugars can be determined by the usual reduction processes. It is more important to determine, however, whether the normal process of fission has taken place. Orban proved the absence of lactose in the acute stages of several intestinal affections of children, and Pfaundler found that the inversion of milk sugar does not take place in marasmic children, thus accounting for the lactosuria that has been reported in such cases.

Extract the dried stool with water; filter; test the filtrate with or without evaporation, with Fehling's, Trommer's test, Nylander's, phenylhydrazin, or Benedict's test. Albumoses and peptones are interfering substances; therefore, it is preferable to extract with alcohol, filter, and evaporate the alcohol, dissolve residue in water, and apply the reduction tests.

Starch may best be determined by the microscope or the microchemical methods already described.

Of pure chemical methods we shall describe the well-known Schmidt fermentation test. This test depends upon the fact that the dissolved carbohydrates, as well as those starches which lie free and are easily acted upon, are inverted by the diastase which is always present in the feces, and are then fermented by the intestinal bacteria, with the formation of gas.

Technic.—

Place 5 grams of stool, well mixed with water, in the receptacle of the Schmidt apparatus, close with a rubber stopper, excluding air bubbles. Connect the outlet tube with a closed graduated tube filled with water, in which the gas that is formed collects. Join this in turn to a tube where the displaced water passes. Fill and set up and place in the incubator at 37° C. for 24 hours. If gas has collected in the tube, cool the apparatus to the temperature of the room and measure the gas. Test the reaction of the fecal mixture with litmus. An acid reaction plus gas indicates the presence of fermentable carbohydrates. Only a positive result is of value.

Schmidt claims that a patient should take a test diet for several days and if the gas fills one-fourth of the tube, intestinal dyspepsia can be proved. Rice, cellulose, and fat should be excluded from the diet.

Concretions.—Our interest in the various kinds of concretions found in stools lies mainly in distinguishing coproliths from real concretions such as gallstones, pancreatic calculi, and intestinal sand. It is comparatively simple to distinguish between a stone made up of organic from one of inorganic structure; heat on a platinum dish causes the organic material to carbonize and crumble away, while the inorganic substance remains unchanged or blackens a trifle. Furthermore, organic pancreatic stones, according to Minnich, on heating give off an aromatic odor and are easily soluble in chloroform.

Gallstones consist of a mixture of the constituents of the fluid in which they are produced. They are always "mixed"; that is, they have more than one constituent. Cholesterin is found in all stones. Calcium salts of the bile pigments are commonly present; sometimes they form the chief part of the stone. Naunyn divided them into the following classes:

(a) *Pure cholesterin stones,* smooth surface, white to yellow, rarely green or brown on the surface, crystalline, oval or spherical, seldom faceted.

(b) *Laminated cholesterin stones* contain calcium salts of the bile pigments, found in yellow, brown, or green layers, alternating with the white layers of cholesterin. Cholesterin forms 75 per cent of the stone. They are often faceted.

(c) *Common gall bladder stones:* Chemically like the former; physically they show an external firm laminated crystalline crust which surrounds a softer pigmented nucleus, frequently containing a cavity filled with a yellow alkaline fluid. They are seldom larger than a cherry. Surface is yellow, sometimes white or brown. They are usually faceted.

(d) *Mixed bilirubin-calcium calculi:* Calcium bilirubin is the chief constituent, but there is also found biliverdin, calcium bilifuscin, oxidized bilirubin, always some cholesterin. These stones are small and occur in two forms, one of waxlike consistency, with round irregular surface; the other hard and brittle, steel gray in color, with a metallic luster.

(e) *Rare forms* are found, to wit, amorphous and incompletely crystalline cholesterin gravel with a nucleus of calcium bilirubin, surrounded by cholesterin, varying in size from a grain of sand to a pea; calcareous stones consisting of calcium carbonate with bilirubin calcium, rare in man, but common in cattle and herbivorous animals; concretions with included foreign bodies such as ligatures, needles, plum stones, around which gallstones are formed.

Test for Stones

We have already noted the test in a platinum dish. Boil two grams of the powdered stone in a flask with distilled water to remove the bile acids and other water-soluble substances. Divide the filtered extract into two parts. (a) Evaporate one to dryness and weigh, giving the weight of the water-soluble material in the amount of dry material used. Incinerate and again weigh, this weight being the quantity of water-soluble ash. Evaporate the other half (b) to dryness, and extract the sodium glycocholate with a mixture of alcohol and ether from the dry residue.

Dry the residue from the water extract and extract with warm alcohol, thus separating the fats along with a small proportion of the cholesterin. Extract the residue from this ether, which dissolves the cholesterin. Evaporate the ether, weigh the cholesterin, and examine for crystals of cholesterin, which are best seen by re-crystallizing from warm alcohol; also try the chemical reactions for cholesterin. Crystals treated with sulphuric acid (5 parts of concentrated sulphuric acid with one part of water) melt at the edges and turn crimson. Add a small quantity of iodine and note a play of colors: violet, blue, green, and red. Evaporate a small quantity on the edge of a porcelain crucible with hydrochloric acid and add a trace of ferric chloride; this gives a blue color. Dry cholesterin dissolved in chloroform and mixed with an equal volume of concentrated sulphuric acid gives, on shaking, a blood red upper layer of chloroform which changes to cherry red and finally to purple, and a lower green fluorescent layer of sulphuric acid. With dilute solutions, the chloroform solution is yellow to rose red, while the sulphuric acid is yellow with a greenish fluorescence. A small quantity of cholesterin, mixed with acetic anhydride, gives a rose color changing to blue and then to blue green on adding concentrated sulphuric acid drop by drop (Liebermann-Buchard reaction). A specimen, purified by repeated crystallization from hot absolute alcohol and dried, melts at 145° C.

After treatment with ether, extract the residue with dilute hydrochloric acid (1 to 3) and filter. (Calcium, if present, is shown by making a portion of the solution alkaline with ammonia and adding acetic acid and ammonium oxalate solution. Copper may be detected by adding a few drops of potassium ferrocyanide, when a brown color develops.) Wash the residue on the filter with water until free from hydrochloric acid. Dry the filter, cut into pieces, and warm in a dry flask with chloroform. Filter and examine for bilirubin, evaporate a small portion spontaneously on a watch glass, showing microscopically small, ill-defined rhombic plates, or indistinct crystalline grains which on being moistened with nitric acid give the colors of Gmelin's reaction (page 1289). Again, shake the chloroform mixture with dilute sodium hydroxide; the pigment goes into alkaline solution, while the chloroform remains decolorized. The separated alkaline solution left to stand in the air turns green from the formation of biliverdin. Detect the presence of hydrobilirubin by the spectroscope.

Examine the stools carefully for stones, especially after washing in a sieve arrangement already described. Many of the objects commonly called gallstones are not real stones; they are intestinal sand coproliths. Chemical and microscopic study of all stones must be made to verify the diagnosis. With the aid of the roentgen ray, too, much information about the gall bladder region is obtainable which will substantiate the stool findings. An artefact is sometimes seen after the administration of large quantities of olive oil in suspected cholelithiasis. It is a concretion composed of fatty acids, fats, and soaps; it is soft, greasy, and transparent. Microscopic and chemical examinations will reveal its true nature.

Pancreatic Calculi are rarely found, less than a hundred cases having been reported. They are small, gray white, with no facets on the surface. Heated on a platinum dish they yield a large amount of white ash and give off an aromatic odor, as above stated. They are soluble in chloroform. Chemically they consist of 50% lime, as carbonates, phosphates, or even oxalates. Pigments and cholesterin in a very small amount are found in the external layers. Cammidge (l. c.) presents a very good illustration of these stones.

Intestinal Sand has been described above.

BACTERIOLOGY OF THE STOOLS

When we apply bacteriologic methods to a study of stools, we are apparently entering upon a task that has no ending. The variety and number of bacteria normally found in stools are so great that ordinarily, unless there is some special reason, we do not make a thorough bacteriologic test. There are certain occasions, however, when a bacteriologic study must be made. Some mathematically inclined bacteriologist has shown that a normal individual passes out via the stool every day some *one hundred and twenty-six billion bacteria,* of many varieties and kinds. What would it avail one, therefore, to conduct routine bacteriologic analyses?

Detailed methods for bacteriologic examination of the feces are found in Chapter X, pages 1629 ff.

In searching for bacteria, feces should be examined as soon after passage as possible. They should be passed in a sterile vessel, placed on ice, and rapidly smeared, stained, plated, and inoculated on special media or placed at once in a preserving medium. Refer to the chapter on Bacteriology. The investigation of the stools of babies is preferably carried out by procuring the stool by means of a specially devised and sterilized glass tube. That suggested by P. Cohnheim is very serviceable. (See Fig. 311, page 1265.)

CHAPTER IX

SPECIAL TESTS

C-REACTIVE PROTEIN*

C-reactive protein is an abnormal protein which appears in blood in the acute stages of various inflammatory disorders and which is undetectable in the bloods of healthy persons. The name of the protein is derived from the fact that it forms a precipitate with the nontype-specific somatic C polysaccharide of the pneumococcus. It is an alpha-globulin and its production is stimulated by bacterial infections, various pyogenic agents (typhoid vaccine), or the products of injured tissue (as in myocardial infarction).

The C-reactive protein was isolated and purified by MacLeod and Avery in 1941.[1] By injecting the purified protein into rabbits, an antiserum is obtained (C-reactive protein antiserum, CRPA). This antiserum is used for the demonstration of C-reactive protein in human serum by the employment of a simple precipitation test.[2]

C-reactive protein is invariably present in the sera of patients with frank rheumatic activity as well as in the sera of patients with low-grade rheumatic activity. The presence of CRP in the serum of a rheumatic patient is an "extremely reliable and sensitive indicator of rheumatic activity." The test is not specific (since CRP appears in blood as a response to other inflammatory conditions also); however, in the case of questionable rheumatic fever, with minimal or no clinical signs, the presence of CRP may be of diagnostic value. The absence of CRP rules out rheumatic activity.

In following the course of treatment in rheumatic fever, the disappearance of CRP from the patient's serum is a true reflection of inflammatory activity as a result of therapy or spontaneous remission.

The advantages of the CRP test over the erythrocyte sedimentation rate are: the sedimentation rate may be elevated without inflammation, as in anemia, pregnancy, convalescent stage of infectious disease, nephrotic syndrome, and hyperglobulinemia; it may be normal in cases with frank rheumatic activity in the presence of congestive heart failure. In such cases, CRP, which is present *only* in inflammatory conditions, is a valuable adjunct or substitute for the sedimentation test.

In rheumatic patients treated with ACTH and cortisone, C-reactive protein reappears upon discontinuing treatment. This is due in most cases to the so-called "rebound phenomenon" which disappears within days. When CRP is present in such cases for a period longer than two weeks, treatment is renewed, since the persistence of CRP is considered evidence of the persistence of rheumatic activity.

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*This section contributed by Alex C. Sonnenwirth, M.S., St. Louis, Mo.

[1] J. Exper. Med. 73: 183, 1941.

[2] Anderson, H. C., and McCarty, M.: Am. J. Med. 8: 45, 1950.

In Sydenham's chorea the CRP test is negative, a positive test being indicative of associated carditis. In cases of coronary insufficiency *without myocardial necrosis,* the CRP test is negative. In cases of myocardial infarction and coronary occlusion and of myocardial infarction with coronary insufficiency, the test is positive.

It is to be expected that studies of CRP in various other inflammatory conditions may increase the value and usage of this test. There are preliminary reports (1955) indicating areas in which the CRP test may be useful (differentiation of widespread malignant disease from primary carcinoma, viral hepatitis, tuberculosis). However, it would be premature at this time to draw general conclusions from these reports, since they have not yet been adequately evaluated.

At the present time the CRP test is accepted as a valuable aid in the diagnosis of low-grade, questionable rheumatic fever, in following the course of this disease during treatment, and in the differential diagnosis of coronary insufficiency, particularly as to the presence or absence of myocardial infarction and cessation of inflammation.

Regular Technic.—

Draw up the CRPA* to one-third of a 90 mm. long, 0.4 mm. inside diameter capillary tube length. Place the finger over the tube. Wipe the tube clean.

Draw an equal amount of the patient's serum into capillary.

Invert the tube several times, slowly, to insure proper mixture of CRPA and serum. Do not let an air pocket form between CRPA and serum.

Insert the capillary tube in the capillary tube rack, making sure that the bottom meniscus of the fluid column in the capillary tube is several mm. (5 to 10) above the clay. Wipe the capillary tube clean to facilitate subsequent reading.

Place the rack in an incubator set at 37° C. for 2 hours. At the end of this time preliminary reading can be made to determine whether the reaction is positive or negative; presence of precipitate indicates a positive reaction.

Remove the rack from the incubator and place in a refrigerator at 40° C. overnight. At the end of this time, a final reading may be made as to the relative quantitative aspect of the reaction. It must be borne in mind that packing may not have occurred. In such cases, where the precipitate is dispersed throughout the fluid column, the reading may be obtained from the summation of the total dispersal precipitate. Semiquantitative as well as definite qualitative readings are best made against a dark background, using oblique illumination.

The readings may be evaluated as follows:

No visible reaction	0
Definite but slight reaction 1 mm. column of precipitate	1+
Moderately strong (2 mm.)	2+
Strong reaction (3 mm.)	3+
Very strong reaction (4 mm.)	4+

Finger-tip Technic.—

A modification of this method has been worked out by Goldin and Kaplan, which is known as the "finger-tip method," which can be used for children and debilitated adults.

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*C-reactive protein antiserum available from Schieffelin & Co., 18 Cooper Square, New York 3, N. Y.

Draw the blood into capillary tubes, larger than those used for the CRP test, preferably blood-collecting tubes.

Seal the tip of the tube with paraffin or in a flame.

After the blood has clotted it may be centrifuged or allowed to stand overnight at room temperature.

The inside diameter of these special tubes should be large enough to allow transfer of the serum directly to the capillaries in which the CRP is being run.

The evaluation of the test is as stated in the regular technic.

DISCUSSION OF HORMONE ASSAYS*

Gonadotropins

Gonadotropins fall into two categories: those of pituitary origin and the chorionic gonadotropin. The biologic characteristics of these substances suggest that they are different chemically. The action of chorionic gonadotropin in human pregnancy urine (PU) simulates that of LH (luteinizing hormone) in the male and FSH (follicle-stimulating hormone) plus LH in the intact female rodent.

The actions of gonadotropins are usually measured by one or more of the following observations in rats or mice: (a) increase in ovarian weight, (b) increase in uterine weight, (c) proportional incidence of vaginal cornification, (d) premature opening of vagina, (e) formation of corpora lutea, (f) changes in the histology of the ovaries, vagina, and uterus, and (g) increase in the weight of the seminal vesicles and prostate.

In interpreting the assays of gonadotropins it is important to take into account that the proportion of FSH and LH affects the qualitative and quantitative responses. These hormones act synergistically and when one of them is present in much larger amounts than the other the characteristic effects of the other may be masked. This is particularly true if a large amount of FSH is present in an extract with a small amount of LH. It is possible for a dosage of an extract containing much LH and little FSH to produce the same size ovaries as a dosage of another extract containing much FSH and little LH. Therefore, a quantitative assay gives little information concerning the amount of each substance present unless it is also accompanied by a careful qualitative analysis.

To determine the approximate amount of the two factors in any given specimen, it is necessary to compare their relative activities by the ovarian and seminal vesicle response. The enlargement of the ovaries is governed chiefly by FSH and seminal vesicle largely by LH, although in each instance the response is increased by the presence of the other factor.

The finding of mature unruptured follicles in immature mice or rats indicates the presence of FSH; hemorrhagic follicles or mature corpora lutea alone or in combination signify LH.

In clinical studies of gonadotropin secretion the effect of a mixture of these hormones is usually determined (gonadotropins, total activity). The gonadotropins are extracted either by precipitation with alcohol or by ultrafiltration. Gorbman[1] described a convenient method for the ultrafiltration of urine through a collodion membrane under pressure. Immature mice are used most frequently for the bio-assay because they are approximately five times as sensitive as rats. It is advisable to use both uterine and ovarian weights as assay end points. With small doses uterine weight increase occurs without ovarian weight increase. At higher dose levels, increase in ovarian weight and maximal increase in uterine weight are observed.

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*Contributed by V. Allen, Director, Biochemical Procedures, Inc., Los Angeles, Calif.

[1] Gorbman, A.: Endocrinology 37: 177, 1945.

Normal male urine so processed and assayed for gonadotropin (in 21- to 24-day-old female mice weighing from 6 to 8 grams) gave values of 5 to 15 mouse uterine units per 24-hour urine sample, a unit being the amount of hormone necessary to produce a 100 per cent increase in uterine weight in immature mice.

Using alcohol precipitation or dialysis extraction technics and the mouse uterine weight, Klinefelter and associates[3] found that normal postpuberal males usually excreted 6.6 mouse uterine units or more per 24 hours; none excreted as much as 105 units. Regularly menstruating females excreted from 6 to 50 mouse uterine units per 24 hours. This laboratory utilizes the alcohol precipitation method with ether and methanol wash, and reports the result in mouse uterine units per 24 hours of pituitary gonadotropin.

Chorionic Gonadotropin.—

Although pregnancy urine of itself increases ovarian weight to a slight extent, studies in hypophysectomized animals show that it does not produce follicular growth and maturation unless some FSH is present. The increased ovarian weight resulting from the two substances is far in excess of their additive effects. The production of luteinization by urinary extracts without doubling the weight of the ovary is an indication of PU (pregnancy urine).

In this country Weisman and co-workers[4a] introduced the use of the South African frog *(Xenopus laevis)*. The mature female Xenopus extrudes eggs within a few hours after the injection of human chorionic gonadotropins. The accuracy of the test in the diagnosis of pregnancy exceeds 99 per cent. This also applies to the use of the North American frog, *Rana pipiens,* which extrudes spermatozoa upon introduction of human chorionic gonadotropin.

There usually is an abnormally large amount of gonadotropins in the blood of toxemic patients; in individuals with hydatid mole or chorioepithelioma it greatly exceeds that of normal pregnancy. If several weeks after a mole is delivered chorionic gonadotropin is present, chorioepithelioma should be seriously considered as one possibility. In males, values under 1,000 mouse units are associated with relatively benign tumors of the testis; 10,000 mouse units or more are found with chorioepithelioma.

Unfortunately, the only scientifically acceptable assay for gonadotropins at present is a biological assay. Chemical or physical (polarographic) methods for the determination of gonadotropins are, to our knowledge and experience, without merit. Therefore, it is our opinion that the accuracy and flexibility of these assays are limited.

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[2] Jungck, E. C., Maddock, W. O., and Heller, C. G.: J. Clin. Endocrinol. 7: 1, 1947.

[3] Klinefelter, H. F., Jr., Albright, F., and Griswold, G. C.: J. Clin. Endocrinol. 2: 529, 1943.

[4] Heller and Heller: Endocrinology 24: 319, 1939.

[4a] Weisman, A. L., Snyder, A. F., and Coates, C. W.: Am. J. Obst. & Gynec. 43: 135, 1942.

Estrogens[5, 6]

Chemical Methods.—

The most obvious and ultimately the definitive method of chemical measurement of presumed estrogens is the weighing of the isolated crystalline compounds and their identification by melting points, melting points of derivatives, and other physical characteristics. The gravimetric determination of estriol in late human pregnancy urine is practical in view of the relatively large amounts obtainable and the possibility of separating this compound out almost quantitatively by fairly simple fractionation and subsequent chromatography. In all other instances in which analysis of human estrogens has been sought, isolation is not practical unless extremely large quantities of starting material are used and ordinarily rather complex extraction procedures employed. In clinical analysis the most common micromethod employed is an estrogen color reaction or fluorometric measurement.

When a solution of a steroidal estrogen is heated in a mixture of phenol and sulphuric acid, cooled, diluted with water, and reheated, a pink color having an absorption maximum at 520 millimicrons develops. Kober, Cohen and Marrian, Venning and associates, and Bachman, among others, have introduced modifications of this procedure with a view to making the reaction more specific and less affected by extraneous chromogens. When solutions of pure compounds are used, the reaction displays a remarkable specificity for the steroidal estrogens. However, in urinary extracts a brown pigment is formed which obscures the pink Kober color. This interference is most marked in urines containing small amounts of estrogens.

The problem of extraneous interfering chromogens in nonpregnancy urine has been attacked in three ways: (1) the quantitative measurement of the material and the application of an appropriate correction, (2) the destruction of the estrogen color, and (3) the purification of the urinary extract by utilization of solubility in various solvents with a view to the elimination of the interfering material.

The observation that phenolic and naphtholic steroids are converted to fluorescent substances by treatment with strong sulphuric acid was made many years ago, but it was only recently that attempts have been made to apply this reaction to the quantitative estimation of urinary estrogens.

Finkelstein and co-workers,[7] Bates and Cohen,[8] and Jailer[9] published photofluorimetric methods for the estimation of estrogens. Finkelstein and associates convert the estrogens to the fluorescent substances by heating with phosphoric acid while the other two groups use sulphuric acid for this pur-

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[5] Stevenson, M. F., and Marrian, G. F.: Biochem. J. **41**: 507, 1947.

[6] Cohen, H., and Bates, B. W.: J. Clin. Endocrinol. **7**: 701, 1947.

[7] Finkelstein, M.: Proc. Soc. Exper. Biol. & Med. **69**: 181, 1948.

[8] Bates, R. W., and Cohen, H.: Fed. Proc. **6**: 236, 1947.

[9] Jailer, J. W.: Endocrinology **41**: 198, 1947.

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Werthessen, N. T., and Baker, C. F.: Endocrinology **36**: 351, 1945.
Wolfe, J. D., Hershberg, E. B., and Fieser, L. F.: J. Biol. Chem. **136**: 653, 1940.
Engel, L. L., Slaunwhite, W. R., Jr., Carter, P., and Nathass, I. T.: J. Biol. Chem. **185**: 255, 1950.

pose. The photofluorimetric methods have the advantage that there is relative to the Kober reaction less interference due to contaminating pigments and fluorophors. This method has been found useful for following estrogen excretion in patients and gives values consistent with bio-assay findings. Its speed, simplicity, and the small volumes of urine required recommend its use as the best and most feasible of the various methods of estrogen determination in the clinical laboratory.

17-Ketosteroids[10-14]

Accumulated evidence indicates that the adrenal cortices and the interstitial cells of the testes secrete steroids which are excreted in part in the urine as 17-ketosteroids. To a limited extent adrenocortical function can be distinguished from gonadal by simple procedures, i.e., the relative proportion of cis (beta) and trans (alpha) stero-isomerism with respect to the hydroxyl at position number 3. The cis (beta) fraction is believed to be derived primarily from the adrenal cortex, while the trans (alpha) fraction is believed to result from hormones elaborated by the gonads. This laboratory uses the method which employs ether extraction accompanied by thorough alkali washing and then followed with the color development with alcoholic potassium hydroxide and meta dinitrobenzene. The fractionation is completed with the use of alcoholic digitonin for precipitation of the beta fraction.

Biological and polarographic assays are also utilized in these determinations.[15, 16]

Corticosteroids[17-20]

The term urinary corticoid or corticosteroids refers to substances of adrenal cortical origin. This laboratory utilizes the method which entails n-butyl alcohol extraction at a pH of 1.0 and consequent purification of extract with color development by way of phenylhydrazine hydrochloride and sulphuric acid. Corrections must be made for reagent color and urinary chromogens and also char development.

Pregnandiol[21-24]

Pregnandiol is the chief urinary transformation product of progesterone in human urine and provides a measure of both luteal and adrenocortical function. Some pregnandiol is also derived from desoxycorticosterone. This

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[10] Drekter, I. J., Pearson, S., Bartczak, E., and McGavack, T. H.: J. Clin. Endocrinol. 7: 795, 1947.

[11] Salter, W. T., Cahen, R. L., and Sappington, T. S.: J. Clin. Endocrinol. 6: 52, 1946.

[12] Masuda, M., and Thuline, H. C.: J. Clin. Endocrinol. 13: 581, 1953.

[13] Pond, M. H.: J. Clin. Endocrinol. 13: 1544, 1953.

[14] Zimmerman, W.: Ztschr. f. physiol. Chem. 233: 257, 1935.

[15] Wolfe, Hershberg, and Fieser: J. Biol. Chem. 136: 653, 1940.

[16] Barnett, Henly, and Morris: Biochem J. 40: 445, 1946.

[17] Heard, R. D. H., and Sobel, H.: J. Biol. Chem. 165: 687, 1946.

[18] Talbot, N. B., Albright, F., Saltzman, A. H., Zygmuntowicz, A., and Wixom, R.: J. Clin. Endocrinol. 7: 331, 1947.

[19] Daughaday, W. H., Jaffe, H., and Williams, R. H.: J. Clin. Endocrinol. 8: 244, 1948.

[20] Reddy, Jenkins, and Thorn: Metabolism 11: 511, 1952.

[21] Bloch, H. S., Zimmerman, B., and Cohen, S. L.: J. Clin. Endocrinol. 13: 1206, 1953.

[22] Sommerville, I. F., Gough, N., and Marrian, G. F.: J. Endocrinol. 5: 247, 1948.

[23] Swyer, G. I. M.: Lancet 256: 104, 1949.

[24] Guterman: J. Lab. & Clin. Med. 33: 256, 1948.

laboratory uses the toluene extraction followed by sodium hydroxide methanol precipitation. Fritted glass filtration and purification by relative solubility then gives a residue which can be developed with sulphuric acid.

LABORATORY TESTS FOR PREGNANCY

Attempts have been made for a number of years to work out a satisfactory test for pregnancy. The need for such a test is sometimes very great; for instance, to determine the presence or absence of ectopic pregnancy, on which the performance of an emergency surgical operation depends. Early pregnancy, too, has been confused with cysts of the ovary and uterine fibroids. Ectopic pregnancy has been confused with pelvic inflammation. The following conditions associated with amenorrhea often mimic early pregnancy.*

1. Lactation and the onset of the menopause.
2. Endocrine dysfunctions, such as ovarian, anterior pituitary, thyroid, and adrenal.
3. Metabolic disturbances frequently associated with obesity.
4. Ovarian cysts and uterine fibroids.
5. Primary and severe secondary anemias.
6. Debilitating diseases, such as tuberculosis and acute infections.
7. Chronic intoxications: lead and alcohol.
8. Psychic amenorrhea due to fear of pregnancy, especially in the unmarried, and the anxiety to bear a child.

After struggling through many different methods of making this diagnosis by laboratory methods, we have finally come to accept the Aschheim-Zondek test or modifications as just the test that is needed for this purpose. These tests depend on the demonstration of a gonadotropic hormone derived from the placenta, similar to the pituitary hormone, in the urine in pregnancy. The test was worked out after it had been previously determined that the anterior pituitary hormone was easily demonstrable in the urine of pregnant women. It is naturally eliminated in the urine of gravid women due to the increased permeability and the low renal threshold observed in pregnancy.

The original technic of Aschheim-Zondek is as follows:

Mouse Method

Aschheim-Zondek Technic

The test is carried out on infantile, three-week-old white female mice that do not weigh more than six to eight grams. Five of these infantile mice are used in the test. Some of the mice are likely to die from the toxic properties of the urine. It is to be noted that the reaction may be positive on the right ovary and negative on the left ovary. The pregnancy is considered positive even though the reaction is present in only one ovary.

It is preferable to obtain the urine by catheter, although this is not essential because these young mice are rather resistant to infection. The urine must be collected in a clean vessel. If it is to be sent from a distance, add to each 25 c.c. one drop of pure tricresol. Shake the urine so that the tricresol is thoroughly mixed. Thirty c.c. of urine are sufficient for the test.

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*Wohl: Bedside Interpretation of Laboratory Findings, The C. V. Mosby Co., St. Louis, Mo.

Before making the test, take the reaction of the urine. If it is alkaline or neutral, add a few drops of acetic acid until the urine reacts acid to litmus paper. If the urine is cloudy, it should be filtered.

The urine is injected in six portions, divided over a period of twenty-four hours. It is preferable to begin the tests on Monday or Tuesday so that they can be completed by the end of the week. The schedule of injection of urine into mice is as follows:

1. Monday morning, between 11 and 12.
   Monday afternoon, 5 P.M.
2. Tuesday morning, 10 A.M.
   Tuesday afternoon, 1 P.M.
   Tuesday afternoon, 5 P.M.
3. Wednesday morning, 10 A.M.

The urine is injected in the following amounts subcutaneously:

Mouse No. 1—six times, 0.2 c.c.
Mouse No. 2—six times, 0.25 c.c.
Mouse No. 3—six times, 0.3 c.c.
Mouse No. 4—six times, 0.35 c.c.
Mouse No. 5—six times, 0.4 c.c.

If this test is started on Monday, the animals are sacrificed on Friday; if started on Tuesday, they are sacrificed on Saturday.

They are sacrificed with illuminating gas or ether and pinned to a cork board. The abdomen is opened and the sexual organs are examined. In most cases, the diagnosis can be made by macroscopic view of the ovaries. In positive cases, the ovaries are large, hyperemic and show the so-called ''blood spots'' or hemorrhagic spots.

Microscopic sections are then made. When positive, note graafian follicles, hemorrhage into the follicles, and corpora lutea.

It is preferable in making the sections to fix the specimens in Zenker solution rather than Formalin, since Formalin tends to shrink the cells and make the diagnosis difficult.

At times, there is a demand for a quicker method than the original method, so that Zondek has devised a more rapid method. This is particularly important in cases in which operation for extrauterine pregnancy may be needed. Zondek has attempted to concentrate the hormone as follows:

Treat 66 c.c. of urine with a small amount of 10% acetic acid until a weakly acid reaction occurs with litmus, filter, and add 40 c.c. of 96% alcohol.

Shake 5 minutes until a flocculent white-yellow precipitate forms. Let this stand one-half hour and then centrifuge.

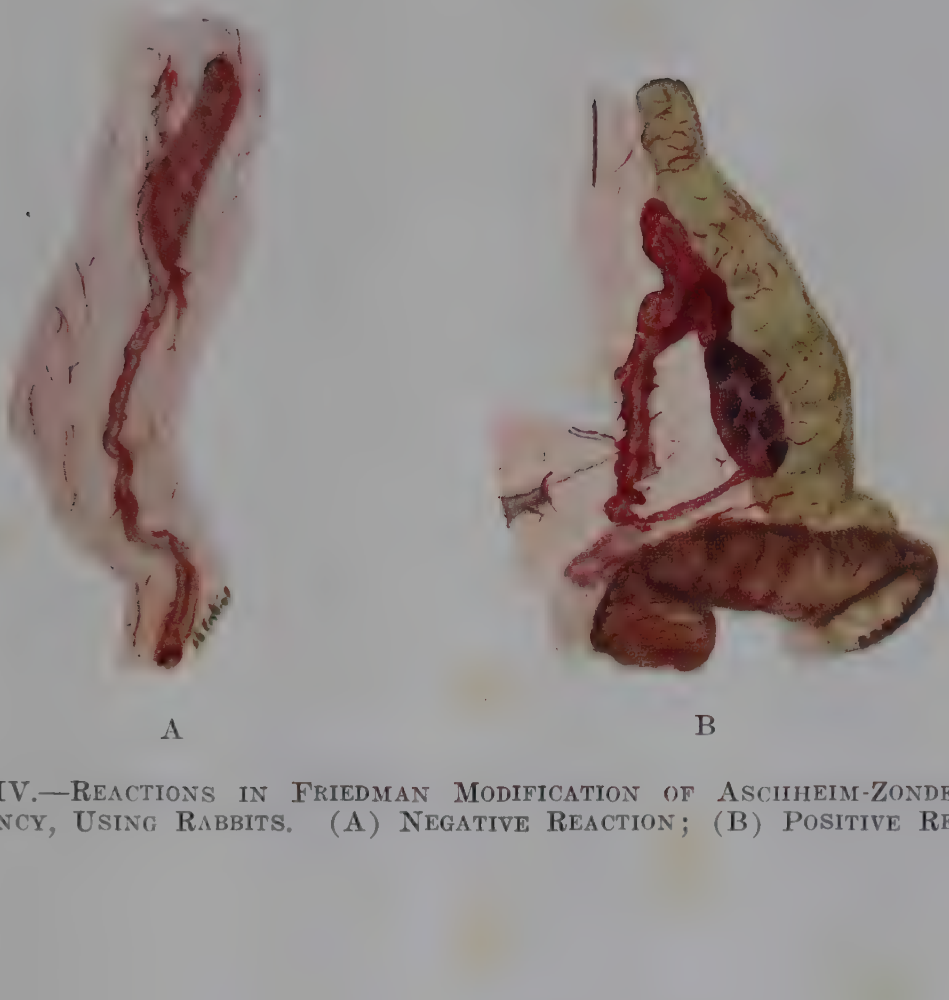
The hormone is in the sediment. Treat this sediment with 30 to 40 c.c. of ether for 3 minutes, shake, pour the ether off, and take up the sediment with 11 c.c. of distilled water, shake 5 minutes, and centrifuge.

The hormone is now dissolved in the water. Throw away the sediment. The solution is yellowish and often becomes cloudy. The whole process takes about 1 hour.

These 11 c.c. of water represent the amount of hormone in 66 c.c. of urine, therefore it is a six-times concentration. Four 6- to 8-gram infantile female mice are each injected every eight hours with 0.4 c.c. of urine six times each, thereby receiving 2.4 c.c. which is equivalent to 14.4 c.c. of the original urine.

After fifty-one and fifty-seven hours, the blood points in the ovary are usually seen so that the diagnosis can be made in the fifty-first or fifty-seventh hour.

Zondek states, however, that the concentrated quick method does not compare in exactitude with the original method, and it is to be used only in



A B

Plate XXXIV.—Reactions in Friedman Modification of Aschheim-Zondek Test for Pregnancy, Using Rabbits. (A) Negative Reaction; (B) Positive Reaction.

emergency cases where it is necessary to make an early diagnosis of extra-uterine pregnancy.

Rabbit Method

Owing to the fact that it is not always possible to have on hand or to secure young female white mice for this test, the use of a young female white rabbit was suggested. We have found rabbits preferable to mice: first, because they are sturdier animals; second, because they are easier to obtain from dealers; and third, because the presence or absence of the blood spots on the ovaries is more easily determined.

The Friedman Modification of the Aschheim-Zondek Test for Pregnancy

The Friedman modification of the Aschheim-Zondek test uses rabbits as ideal test animals for hormone reactions for pregnancy. Although ova continually mature and graafian follicles ripen in the ovaries of rabbits, ovulation does not take place until after copulation. Thus in properly caged female rabbits it is possible to study the effect of urine on ovaries previously free of corpora hemorrhagica. Another reason that rabbits are ideal animals is that after reaching maturity they are always ready for use in the test. One need be assured only that the rabbit has not recently copulated, is not pregnant, or has not been used as a test animal in the previous six weeks or two months.

Technic.—

Method Using Urine.—

The animals to be used are healthy female rabbits seventeen weeks old. On the average, female rabbits attain sexual maturity in five months. If the animal is purchased from an outside source it is caged separately for two weeks before use. Since the period of gestation in the rabbit is only 21 days, pregnancy in an animal will be easily detected by palpation and eliminated at the end of two weeks, or if ovulation without conception has resulted from excitation by a male rabbit, the corpora hemorrhagica will have entirely disappeared by this time.

The urine specimens preferred are the first voided on rising, because the hormone as well as other constituents will be more concentrated in the high-specific-gravity urine voided at this time. The specimen should not be more than four hours old, although evidence is accumulating that the hormone is not so labile as it was formerly believed. It should be received under all circumstances into a sterile bottle. It is preferable, if possible, to withdraw it with a sterile catheter to prevent bacterial contamination. This is especially true for specimens from a distance.

The first injection of 10 c.c. of urine is made into one of the marginal ear veins of a rabbit. The second injection of 5 c.c. is made 24 hours after the first injection, in the same manner. Rabbit should be labeled and kept in separate cage after injections. The ovaries of the rabbit are examined by laparotomy or by anesthetizing the rabbit to death 24 hours after the second injection. There is no difficulty in finding the ovaries of the rabbit or in recognizing a positive reaction.

The reading of the test is not difficult. The negative test shows a white or light pink ovary which is usually studded with clear mature graafian follicles. The uterus is usually not injected. Congestion and injection of the uterus, however, are thought to be produced by the ovarian hormone independent of the anterior pituitary and thus have no significance in the reading of the test.

Positive reactions are seen as follows: ovaries studded with from 1 or 2 to half a dozen or more corpora hemorrhagica and a coiled hyperemic uterus. The small rosy spots that may appear in large clear follicles are suggestive but not positive and another test should be made.

Positive reactions occur in cases in which embryonal tissue is in biological contact with maternal or host tissue. Conditions other than pregnancy in which positive reactions occur are hydatidiform mole, chorioepithelioma, and certain embryonal malignant tumors of the testis. Tubal pregnancy in which the fetus is dead but the placental tissue is still viable, and incomplete abortion with retention of viable placental tissue also give positive reactions.

False negative reactions occur with pregnancies in which all placental tissue is dead, missed abortions, and ectopic pregnancies. They may also be occasioned by the use of an immature animal which is incapable of reacting or by accidental confusion or willful substitution of specimens by persons interested in inducing the physician to curette the uterus in the patient being studied.

Increase in gonadotropic factors reaches the peak between the 30th and 50th days in normal pregnancy. In hydatidiform mole and chorioepithelioma these gonadotropic factors are elevated above the level reached in normal pregnancy. A quantitative test is of importance in the diagnosis of hydatidiform mole because as many as 300,000 mouse units per liter may be present. In chorioepithelioma the hormone amounts vary around 100,000 mouse units per liter. In using this test in chorioepithelioma excluding an early pregnancy, repetition of the quantitative test must be made. The test is useful, too, after operations for chorioepithelioma, to detect metastases.

Method Using Blood Serum.—Female rabbits, ten weeks to three months old, are used. No rabbit is used if it is older than three months. Obtain serum in the usual manner. Inject 2.5 c.c. of the serum intravenously into the marginal ear vein of a rabbit (page 2038). After twenty-four hours, sacrifice the rabbit with chloroform anesthesia, autopsy, and examine as above for corpora hemorrhagica. Refer to the test, using male frogs, page 1317.

Tests for Pregnandiol

Progesterone, which is elaborated by the corpus luteum and placenta, is excreted in the urine as a pregnandiol-glucuronic acid conjugate. Guterman[1] reported a modification of the Astwood and Jones[2] procedure by adding the color reaction of concentrated sulphuric acid on pregnandiol originally described by Kober.[3] Guterman's method shortened the Astwood and Jones procedure to three hours by making only one extraction with toluene and one precipitation of pregnandiol from acetone. Parks and Mack[4] have modified the Guterman procedure so that the test can now be completed in from one to one and one-half hours with an accuracy which they claim surpasses that of the complete Guterman technic.

Guterman Technic.[1]—

A. Hydrolysis and Extraction of Pregnandiol.—

1. Place 100 c.c. urine, first morning specimen,
   50 c.c. toluene, c.p.,
   10 c.c. concentrated hydrochloric acid,

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[1] Guterman, H. S.: J. Clin. Endocrinol. **4**: 262-267, June, 1944.
[2] Astwood, E. B., and Jones, G. E. S.: J. Biol. Chem. **137**: 397-407, Jan., 1941.
[3] Kober, S.: Biochem. Ztschr. **239**: 209-212, 1931.
[4] Parks, A. E., and Mack, H. C.: Harper Hosp. Bull. **4**: 167-168, Sept., 1946.

and 2 glass beads
in a 500 c.c. flat-bottomed Florence flask.

2. Connect the flask via a one-holed cork stopper to a vertical, water-cooled, 400 to 500 cm. jacket-length Liebig condenser.

Boil vigorously over an electric hot plate for 15 minutes.

3. Cool to room temperature under running tap water.

4. Transfer to a 500 c.c. separatory funnel and draw off the lower (urine) layer.

5. Wash the toluene layer and the toluene-water emulsion with 15 c.c. portions of N/10 sodium hydroxide, then twice with 15 c.c. of distilled water.

B. Precipitation of Impurities.—

1. Transfer the washed toluene and toluene-water emulsion to a 125 c.c. Erlenmeyer flask with 2 glass beads.

2. Boil over an electric hot plate (under the hood).

3. When the water has evaporated and the toluene mixture is boiling smoothly, add 10 c.c. of 2% sodium hydroxide in absolute methanol.

4. Evaporate to one-half the original volume of toluene.

5. Filter the toluene mixture while hot through a fritted glass filter (medium porosity, Pyrex) using mild suction. If the filtrate has an orange, pink, or brown tint, steps 3, 4, and 5 must be repeated until the filtrate is yellow or yellow green.

6. Wash the precipitate with 15 c.c. of hot toluene, and combine this washing with the filtrate obtained in the previous step.

7. Evaporate to dryness over the hot plate, in the hood, using a gentle air stream to drive off the last traces of toluene. This prevents charring of the residue.

C. Precipitation of Pregnandiol.—

1. Add 5 c.c. of acetone to the residue (in the previous section) and warm the mixture over a hot plate until solution is complete.

2. Add 20 c.c. of N/10 sodium hydroxide slowly while the mixture is still on the hot plate.

3. Place the flask in an ice-water bath for 30 minutes or in a refrigerator at 5° C. for one hour.

D. Isolation of Pregnandiol.—

1. Use the mixture obtained in section "C-3" and filter through a fritted glass filter of medium porosity, Pyrex, using mild suction.

2. Wash the precipitate with 15 c.c. distilled water.

3. Change the receiving flask and pass 10 c.c. of hot absolute alcohol through the fritted glass filter to dissolve the precipitate.

4. Evaporate the alcohol filtrate to dryness over an electric hot plate in the hood.

E. Color Development.—

Add 10 c.c. concentrated sulphuric acid to the residue and observe the color in a test tube when solution is complete. Colorless to light yellow solution is negative. Orange to deep orange-brown is positive.

Pregnandiol Precipitation Test

Method of Mack and Parks[1]

Repeated attempts have been made to perfect a chemical test for the early diagnosis of pregnancy through identification of pregnandiol in the urine of amenorrheic women. Various methods have been tried, all of which present many technical difficulties. A greatly simplified technic has been developed by Mack and Parks.

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[1] Mack, H. C., and Parks, A. E.: J. Clin. Endocrin. 2: 351, 1947.

Reagents.—

Toluene.—Reagent quality.

Hydrochloric Acid.—Concentrated.

Glass Beads.—

0.1N Sodium Hydroxide.—See pages 25 ff.

2% Sodium Hydroxide in Methyl Alcohol.—

Dissolve 2 gm. of sodium hydroxide, c.p., in absolute methanol and dilute to 100 c.c. with absolute methanol.

Acetone.—

Technic.—

(A) **Hydrolysis and Extraction of Pregnandiol.**—

Secure a first morning urine specimen and record the specific gravity.

Measure 100 c.c. of urine and pour it into a 500 c.c. Erlenmeyer flask.

Add 50 c.c. of toluene
and 10 c.c. of concentrated hydrochloric acid
and 2 glass beads to the flask.

Connect the flask to a vertical Liebig condenser and allow the sample to reflux on a hot plate for 15 minutes.

Remove the flask and cool rapidly to room temperature under a water tap. Avoid agitation of the sample while cooling to prevent formation of an emulsion.

Transfer the sample to a 500 c.c. separatory funnel.

Draw off and discard the lower layer (urine).

Small amounts of emulsion present at this point can be disregarded. If large amounts are present, transfer the sample to a 100 c.c. tube and centrifuge for 5 minutes. The toluene and associated urine are then poured into the separatory funnel, leaving the emulsion in the tube.

Add 15 c.c. of N/10 sodium hydroxide to the separatory funnel and wash by inverting twice and swirling the sample gently.

Discard the sodium hydroxide which settles to the bottom of the funnel.

Repeat by adding 15 c.c. of N/10 sodium hydroxide; then proceed as above, following the same procedure with two 15 c.c. portions of water.

After the last washing with water, make a very careful separation and transfer only the toluene layer to a dry 125 c.c. Erlenmeyer flask.

(B) **Precipitation of Impurities.**—

Add 2 glass beads and bring the sample to boiling on a hot plate.

When the vapors reach the mouth of the flask, slowly add 10 c.c. of 2% sodium hydroxide in absolute methanol while the flask remains on the hot plate.

Boil until the methanol has evaporated and the toluene is reduced to about one-half its original volume.

Filter, while hot, through a fritted glass filter, Pyrex, medium porosity, using suction.

Collect the filtrate in a large test tube.

Add 15 c.c. of toluene to the flask as a rinse, heat to boiling, and use it to wash the precipitate remaining on the filter.

Pour the filtrate from the test tube into a dry 125 c.c. Erlenmeyer flask and evaporate to dryness on a hot plate. Use a gentle air stream to remove the last traces of toluene.

(C) **Precipitation of Pregnandiol.**—

Add 5 c.c. of acetone to the residue in the flask.

Place the flask containing the acetone on a hot plate. When it begins to boil, add 25 c.c. of boiling N/10 sodium hydroxide slowly, 3 to 4 c.c. at a time.

Remove the sample from the hot plate and read.

(D) **Reading of Result.—**

Swirl the sample and observe the side of the flask above the level of the liquid, standing in front of a window or a good source of light. In samples containing 0.75 mg. or more of pregnandiol, a precipitate is evident immediately. Due to surface tension of the liquid and the lightness of the precipitate, it is carried up the side of the flask and becomes evident as tiny, white particles.

If no precipitate is immediately evident, place the sample in an ice-water bath for 10 minutes.

Remove from the ice bath and allow it to stand for 30 minutes at room temperature.

Again observe as above.

As a method of checking the amount and character of the precipitate, follow the procedure for reading and grading Kahn tests.

Place 1 c.c. of the sample in a standard Kahn tube and view it in a fluorescent light box. The reaction can thus be graded as 0, +, ++, +++, or ++++.

(E) **Description of the Precipitate.—**

The substance which is precipitated under the conditions listed above is manifestly impure pregnandiol, contaminated by cholesterol and other steroids. The precipitate is birefringent and does not form clearly defined crystals.

In examination of urine in 94 pregnant and nonpregnant controls and in 121 unknown urine samples, all known pregnancy control urines gave positive tests. Negative pregnandiol tests were obtained in all nonpregnant controls except for determinations made during the luteal phase of the normal menstrual cycle. It may be concluded that false positive pregnandiol tests for pregnancy may be obtained during delayed luteal phases of prolonged cycles where late ovulation has occurred. This limits the accuracy of the pregnandiol test which is most reliable in amenorrhea of otherwise normally and regularly menstruating women.

The test has been applied in serial determinations in 14 instances of threatened abortions. The clinical outcome coincided with laboratory findings in 13 cases. Consistently negative pregnandiol tests foreshadowed abortion in 7 cases; consistently positive pregnandiol excretion or negative tests which became positive coincided with retention of the pregnancy in 6 cases. In 1 instance of abortion at five and one-half months, strongly positive qualitative tests were obtained.

This test suggests its practical usefulness as a rapid means of diagnosis of pregnancy and as an adjuvant to the management of threatened abortion.

The Male Frog Test (Rana Pipiens) for Pregnancy

The gametokinetic response of the male Batrachia to gonadotropic substances was described by Rugh in 1937.[1] The clinical application of this observation to the laboratory diagnosis of pregnancy was reported by Galli-Mainini.[2] Impetus was lent to the popularization of the male frog test for pregnancy in this country by Robbins and Parker.[3] This has been accepted therefore as a rapid, economical, and accurate procedure in the diagnosis of pregnancy. Blood serum was substituted as a test material and has been used by Haskins and Sherman[1] exclusively in routine pregnancy testing. Their opinion and that of others suffice to recommend this male frog test as the best method of blood serum testing for pregnancy, especially in view of the fact that concentration of chorionic gonadotropin is greater in serum than in urine. This method obviates the annoyance and expense of repeating tests because of the premature death of the test animal from toxic substances in urine.

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[1] Rugh, R.: Proc. Soc. Exper. Biol. & Med. 36: 418, 1937.

[2] Galli-Mainini, C.: Semana méd. 54: 33, 1947.

[3] Robbins, S. L., and Parker, F., Jr.: Endocrinology 42: 237, 1948.

Technic.—

Use male *Rana pipiens* obtained from a single laboratory supply house. Sexing of the frogs can be determined by examination of the thenar eminences, which in the male are hypertrophied.

Keep the frogs refrigerated at 6° C. prior to their use; this allows prolonged storage at low metabolic level and obviates the need for feeding them. In other words, purchase a supply of frogs and keep in a refrigerator at 6° C. until time to use them. Refrigeration also reduces the stimulation of the frogs to gametokinesis by amplexus.

Prior to injecting the test animal, examine its urine for sperm. Discard for injection purposes those rare animals in which urinary sperm is found.

Administer 3 c.c. of serum to each of 2 frogs in routine testing, or, better, use 1 c.c. of serum to each 10 gm. of frog weight.

After injection, place each frog in a separate container for the remainder of the test.

Inject the serum intraperitoneally.

Obtain urine from the cloaca by a small pipette, each 30 minutes, until a positive response is obtained, or until 2 hours have elapsed.

Report a test positive when urinary sperm is found in either or both frogs.

This method of testing for pregnancy is especially recommended. In 800 frog tests performed by Haskins and Sherman, accuray in the test was 97 per cent.*

The "Frog" Test (Xenopus Laevis) for Pregnancy†

Weisman, Snyder, and Coates[2] have given ample evidence of the usefulness of the frog test in the rapid diagnosis of pregnancy. This test uses the South African clawed frog, *Xenopus laevis.* The advantages of this test are its high degree of accuracy and the rapidity of reaction, i.e., four to eighteen hours. Weisman and associates report their findings in 53 clinical cases of suspected pregnancy.

This frog test is based on the peculiar biologic function of the mature female Xenopus in that she carries eggs throughout the year, extruding them only at mating or after the injection of hormones peculiar to pregnancy. Since the test animals are kept isolated from the males, the extrusion of any ova following the injection of suspected urine becomes definitive for pregnancy. Parallel tests on segregated rabbits were used by Weisman and associates[3] on the

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**Rana pipiens* may be secured from E. G. Steinhilber & Co., Oshkosh, Wis. This company ships frogs every day. If an order is received in the late afternoon, they are shipped the following morning, Monday through Thursday. Saturday and Sunday deliveries are avoided. Most shipments are made by air parcel post or surface parcel post, rather than by express, to save transportation costs and speed delivery. Shipments for Monday delivery are made ordinarily on Saturday by air parcel post. To be sure of fresh stock, order often during the summer months. To be sure of a steady supply, the supplier recommends placing a standing order for a specified number to be shipped or delivered at a certain time each week. One's requirements thus are scheduled regularly, and deliveries are made without having to reorder each time. In ordering, specify "special males."

†*Xenopus laevis* frogs may be procured from Jay E. Cook, Cockeysville, Md. See also Weisman and Coates' bulletin entitled "The South African Frog (Xenopus Laevis) in Pregnancy Diagnosis," publisher, New York Biologic Research Foundation, 1944.

[1] Haskins, A. L., and Sherman, A. I.: Am. J. Obst. & Gynec. **61**: 5, 1951.

[2] Weisman, A. I., Snyder, A. F., and Coates, C. W.: Am. J. Obst. & Gynec. **43**: 135, 1942.

[3] Weisman, A. I., Snyder, A. F., and Coates, C. W.: West. J. Surg. Obst. & Gynec. **50**: 557, 1942.

same specimens of urine as they used on the frogs. In one case of disagreement, where the frog test was positive and the rabbit test was negative, the patient was operated upon with a preoperative diagnosis of ectopic pregnancy. Upon laparotomy, an unruptured tubal pregnancy was discovered in the pelvis, the diagnosis of which was later confirmed in the pathologic laboratory. In this instance, the frog test was proved to be correct and the rabbit test false. These investigators believe that the frog test is a more sensitive indication of early pregnancy than the rabbit test. Table 134 of Weisman and associates shows the advantages of this frog test.

TABLE 134

		XENOPUS	A-Z (MICE)	FRIEDMAN (RABBIT)
1	Rapidity	4-18 hr.	96 hr.	36-48 hr.
2	Simplicity	No operation	Requires operation	Requires operation
3	Economy	$6.00 per frog*	5 mice cost $2.50	One rabbit $2.50
4	Maintenance	Simple	Feeding and cleaning problem	Space occupying
5	Technic	Single injection	40 injections	One or two injections
6	Ease of injection	Subcutaneous	Multiple injections	Intravenous
7	Practicability	Can be used over and over again	Animals killed	Rabbit usually killed
8	Special precautions	None	Age and weight	Isolation
9	End reaction	Simple observation of eggs in water	Search for ovaries in five animals	Search for ovaries

*Frogs are used over and over again.

Technic.—

The basic method of keeping the frogs entails the use of three twelve-gallon aquaria for each group of eight frogs. Tank *A* is used as a "rest" tank in which the animals recuperate for four weeks after having extruded ova in a positive reaction. Tank *B* is the "negative reaction" tank in which those frogs not reacting to injections of urine (negative test) are rested for a week. Tank *C* is the "active" tank from which animals ready for use are taken. It is apparent that both *B* and *A* become "active" tanks when the rest periods are completed.

Water in the tanks is kept at ordinary room temperature, 70° F., and is maintained at a level of about three inches from the bottom. The frogs are fed small strips of beef heart, calf liver, and garden worms (if available) twice each week. Tank water should be replaced by fresh water of the same temperature prior to each feeding and 24 hours after each feeding. The animals should not be fed for at least 24 hours prior to testing since the injection causes regurgitation of partially digested food which may interfere with the visualization of the eggs and the correct reading of the test.

For the actual tests, standard two-gallon fish tanks are used. These are stacked one above the other if a number of tests are to be made simultaneously. Half-inch wire mesh should be bent to form a platform about one inch from the bottom of each test tank to prevent the frog from devouring its own extruded eggs. A set-up test tank should contain about three inches of water and should be kept covered with a glass plate, slightly elevated above the frame of the tank to allow passage of air. The glass should be taped or weighted down to the tank so the frogs cannot climb out. The water in all the tanks should be of the same temperature and the room for housing and testing should be bright and airy with little variation in temperature. After each test it is advisable to burn off any contaminating eggs from the wire platform with a Bunsen burner.

HORMONE

CHORIONIC GONADOTROPIN	PITUITARY GONADOTROPIN	17-KETOSTEROIDS
NORMALS: **Normal Female:** Trace amounts **Pregnant Female:** Increases within first two months to peak of 100,000 and more mouse uterine units/24 hr., decreasing after 8th-12th wk. to 2,000-10,000 mouse uterine units/24 hr.	**NORMALS:** **Male and Female:** 6-50 mouse uterine units/24 hr. **Male Children:** Under 11 yr.: 0 **Female Children:** Detectable in small amounts one year prior to menstruation	**NORMALS:** **Female:** 6-18 mg./24 hr. **Male:** 10-22 mg./24 hr. **Children under 6 yr.:** Less than 1 mg./24 hr. increasing gradually to age 18; normal adult range may be encountered after age 12 **After 60 yr. of age:** Male: 2-9 mg./24 hr.; female: 1-8 mg./24 hr. **Normal Beta/Alpha Ratio:** Approximately 1/10-1/9
PATHOLOGIC FINDINGS: **Increased in:** Testicular tumors (mixed epithelioma type) Hydatidiform mole Chorioepithelioma	**PATHOLOGIC FINDINGS:** **Increased in:** Cushing's syndrome Hyperpituitary activity Menopause Seminiferous tubule failure Male climacteric **Decreased in:** Simmonds' disease Panhypopituitarism Administration of excessive amounts of testosterone and estrogens	**PATHOLOGIC FINDINGS:** **Increased in:** Testicular tumors (interstitial cell)—very high Adrenal hyperfunction Following endocrine therapy (ACTH, testosterone, chorionic gonadotropin) Severe stress Human pregnancy (third trimester) Ankylosing spondylarthritis Cushing's syndrome Simple hirsutism **Decreased in:** Female hypogonadism, moderately low if associated with various metabolic disorders. Normal in majority of patients with primary ovarian failure, male hypogonadism, pituitary tumors, thyrotoxicosis, hypertension, diabetes mellitus, Addison's disease, hypothyroidism, nephrosis, gout, chronic illnesses. Acute illnesses: common cold, malnutrition, anemias, anorexia nervosa, hepatic disease, physical fatigue **Male:** Two-thirds of the total 17-ketosteroids are from the adrenal cortex and one-third from the testes **Female:** Traces from the ovary and the rest from the adrenal cortex **Ratio of Beta/Alpha:** 1/1 is usually evidence of tumor
ANALYSIS REQUIREMENTS: 1. Collect 24-hr. urine specimen 2. Preserve with 5 c.c. toluene 3. Record total volume of urine 4. Send 200 c.c. aliquot with 5 c.c. toluene and note total volume	**ANALYSIS REQUIREMENTS:** 1. Collect 24-hr. urine specimen 2. Preserve with 5 c.c. toluene 3. Record total volume of urine 4. Send 200 c.c. aliquot with 5 c.c. toluene and note total volume	**ANALYSIS REQUIREMENTS:** 1. Collect 24-hr. urine specimen 2. Preserve with 5 c.c. toluene 3. Record total volume of urine 4. Send 200 c.c. aliquot with 5 c.c. toluene and note total volume

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CHART*

ESTROGENS	PREGNANDIOL	CORTICOSTEROIDS	PROTEIN-BOUND IODINE
NORMALS: **Male:** 2-29 μg/24 hr. **Female:** Normal menstrual cycle—4-160 μg/24 hr. **Pregnancy:** Up to 45 mg./24 hr. **Children:** Very small amounts	**NORMALS:** **Normal Menstrual Cycle:** 0-10 mg./24 hr. **Pregnancy:** 50-120 mg./24 hr. **Male:** 0 **Normal Children:** 0	**NORMALS:** **Adults:** 1.0-2.5 mg./24 hr. **Children:** Trace amounts may be found until adult	**NORMALS:** **Adults:** 4-8 μg %
PATHOLOGIC FINDINGS: **Increased in:** Precocious menarche Granulosa cell tumor Hyperplasia of adrenal cortex Tumor of adrenal cortex Theca cell tumor **Decreased in:** Simmonds' disease Panhypopituitarism (milder forms) Following death of fetus Male castrate Diabetic pregnancy Preeclampsia and eclampsia Lutein cell tumor Arrhenoblastoma	**PATHOLOGIC FINDINGS:** **Increased in:** Cushing's syndrome Adrenal cortex tumors Corpus luteum cysts **Decreased in:** Threatened abortion Intrauterine fetal death Toxemias of late pregnancies Dibetic pregnancy	**PATHOLOGIC FINDINGS:** **Increased in:** Pregnancy (1st trimester) Severe hypertension Virilism Surgery Burns ACTH administration (40 mg./24 hr.) Cushing's syndrome Pancreatitis (very high) Eclampsia (very high) **Decreased in:** Addison's disease Anterior pituitary hypofunction	**PATHOLOGIC FINDINGS:** **Increased in:** Hyperthyroidism **Decreased in:** Hypothyroidism
ANALYSIS REQUIREMENTS: 1. Collect 24-hr. urine specimen 2. Preserve with 5 c.c. toluene 3. Record total volume of urine 4. Send 200 c.c. aliquot with 5 c.c. toluene and note total volume	**ANALYSIS REQUIREMENTS:** 1. Collect 24-hr. urine specimen 2. Refrigerate during collection 3. No preservative 4. Send 200 c.c. aliquot with total volume notation	**ANALYSIS REQUIREMENTS:** 1. Collect 24-hr. urine specimen 2. Preserve with 5 c.c. toluene 3. Record total volume of urine 4. Send 200 c.c. aliquot with 5 c.c. toluene and note total volume	**ANALYSIS REQUIREMENTS:** 4 c.c. of plasma or serum

Concentration of Urine.—

The present method recommended by Weisman et al. means that a simple concentration can be made in half an hour or less. Four ounces of morning urine are required, of which 80 c.c. are used. To 80 c.c. of urine, add twice the volume of acetone. Thoroughly stir the mixture and allow to stand for 15 minutes or thereabouts when the precipitated proteins and hormones will have settled to the bottom. Decant the supernatant fluid and save for redistillation. Allow the precipitate to dry. This may be hastened by a current of air induced by a suction apparatus or an electric fan. Add 2 c.c. of distilled water to the dry precipitate and stir thoroughly then pour the mixture into a small centrifuge tube. Centrifuging brings down the insoluble proteins and other material while the gonadotropic hormone remains soluble in the water. Immediately prior to injection, decant the supernatant fluid and adjust with ten per cent sulphosalicylic acid to pH 5.5, using nitrazine paper, Squibb. Use 1 c.c. of the concentrate for each injection, the remaining cubic centimeter being reserved for use in case recheck is necessary; or a second frog may be injected immediately. If you have plenty of frogs, it is better to use two frogs for each test. Each c.c. of the final concentrate is equivalent to 40 c.c. of whole urine.

The Test Animal.*—Only mature, female, healthy and well-nourished animals can be used. Standardized animals—those which have reacted previously by egg extrusion after injection of gonadotropic substance—must be used to attain the greatest possible degree of accuracy.

Mode of Injection.—Select the dorsum of the animal as the site of injection since it is the largest lymph space. Care as well as experience is required when injecting the frog. Puncturing the lung usually means death of the animal. The frog is injected subcutaneously by means of a short insulin-type needle. The lymph space is reached by thrusting the needle superficially into the left thigh muscle, directing the point medially to the midline of the dorsum approximately one-half inch above the upper cloacal fold. Throughout its course, the needle should be seen clearly just beneath the skin. A firm gentle thrust carries it through connective tissue into the lymph space. Do not permit the point of the needle to advance cephalad toward the lungs. If the needle is anywhere near the lung, a sudden effort by the animal may cause lung puncture.

Reading the Test.—After the animals are injected with the urine concentrate, place them in separate waiting "test tanks" and observe at intervals after the first four hours. Positive extrusion of eggs may be seen as early as four hours after injection, but ordinarily the reaction occurs between six and twelve hours. If after 18 hours no eggs are visible in the tank, the test may be considered negative and the animal is placed in tank *B* for a rest of one week.

Six-Hour Pregnancy Test

(Salmon, Geist, Salmon, and Frank)

A 6-hour pregnancy test, using immature white rats, has been originated by Salmon, Geist, Salmon, and Frank.[1]

For the performance of the test, three white rats (35 to 45 gm., ages 22 to 25 days) are injected subcutaneously with 2 c.c. of the first morning specimen and autopsied at the end of six hours.

Ovarian Reaction.—The ovaries are enlarged and bright red in appearance in sharp contrast to the small, white or faintly pink ovaries of the control animals. The administration of human pregnancy urine to immature female rats results in marked hyperemia of the ovaries. That this vascular reaction is due to the gonadotropic factor present in pregnancy urine was shown by the production of a similar vascular reaction with chorionic gonadotropin and by the failure to induce the reaction with pregnancy urine, in which the gonadotropin had been inactivated by boiling. The work of these authors reveals further that urine of pregnant women contains estrogens in such high concentration that administration of 2 c.c. of urine to an immature rat causes proliferation of the epithelial and muscular coats of the vagina, which is detectable microscopically and grossly 24 hours after the injection. The proliferative changes in the vaginal mucosa are identical to those induced by estrogens.

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*Frogs are obtainable from Jay E. Cook. Cockeysville, Md.

[1] Salmon, Udall J., Geist, Samuel H., Salmon, A. Austin, and Frank, Irving L.: J. Clin. Endocrinol. 2: 167-170, 1942.

If, for any reason, it is inconvenient to examine the ovaries at 6 hours, the animals may be autopsied at any time during the following 24 hours, but the ovaries must be examined immediately after death in order to detect the hyperemia of the ovaries. The method used by the authors is to asphyxiate the animals with illuminating gas. In performing the autopsy, it is important to avoid injuring vessels in the abdominal wall, since blood in the abdomen makes it difficult to detect the ovarian hyperemia.

This 6-hour test has the advantage of being more rapid and less costly than the Aschheim-Zondek test performed on five mice, which takes 96 hours, and the Friedman test, which requires 48 hours.

Explanation of This Reaction.—The failure of the pregnant woman to react is explained on the assumption that early in pregnancy the production and absorption of the active substance from the breast leads to an immune state which prevents the local reaction at the site of injection, except the weak reaction seen in 80 per cent of the cases in the first six and the last three weeks of pregnancy, during labor, and in the early days of the puerperium.

URINARY 17-KETOSTEROIDS*

Human urine contains a variety of steroid compounds, acidic, phenolic, or neutral. The acidic steroids represent the bile acids; the phenolic include the estrogens; and the neutral represent principally metabolites from the testes or ovaries and from the adrenals. The term ''17-ketosteroids'' is used with reference to the neutral urinary steroids, which are characterized by a carbonyl linkage at carbon 17. The 17-ketosteroids represent excretion products of hormones coming from the testes of the male and the adrenal cortices of both sexes. Determination of the 17-ketosteroids is made to consider the combined activity of the adrenal cortex and the testes in the male, and of the adrenal cortex alone in the female. Since both the adrenal cortex and the testes are under control of the pituitary, the test may also be considered an indirect measure of the function of that gland.

The principal members of the 17-ketosteroid group are androsterone, isoandrosterone, dehydroisoandrosterone, and etiocholanolone. The 17-ketosteroids are further subdivided into alpha and beta fractions. The beta-17-ketosteroids are precipitated by digitonin and the alphas are not. The alpha-17-ketosteroids normally comprise about 85 to 95 per cent of the total, making the beta : alpha ratio about 1 : 9.

The test, a chemical one with measurable colorimetric end point as a result of the Zimmermann reaction, is considerd as an index of androgenic function. Results parallel the androgen excretion as measured biologically by the effect on the size of the comb of a capon.

The method is that of Holtorff-Koch, a modification of the Zimmermann technic. The normal by this method is 8 to 14 mg.[1]

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*Much of this section is from Roberto F. Escamilla, "Laboratory Aids in Endocrine Diagnosis," Springfield, Ill., 1954, Charles C Thomas. By permission of the author and the publisher. Sections are from Bull. Lab. Med. 1: 8, 1952.

[1] Drekter, I. J.: J. Clin. Endocrinol. 7: 795, 1947.

Modified Method of Holtorff and Koch[2]

Reagents.—

Concentrated Hydrochloric Acid.—

50% Sulphuric Acid.—

Carefully add 50 c.c. of concentrated sulphuric acid, c.p., to 50 c.c. distilled water. When cool, dilute to 100 c.c. with distilled water.

2% Thymol in Glacial Acetic Acid.—

Use 2 gm. thymol per 100 c.c. of glacial acetic acid.

Ether.—

Detergent.—

Use a detergent such as Vel or Dreft.

Sodium Hydroxide-Sodium Hydrosulphite Solution.—

This must be freshly made.

Add 10 gm. of sodium hydrosulphite powder to 100 c.c. of 10% sodium hydroxide solution.

Absolute Alcohol.—

80% Alcohol.—

Metadinitrobenzene Solution.—

This must be prepared fresh for the test.

Dissolve 34.5 mg. of purified metadinitrobenzene crystals[3]
in 3 c.c. of absolute alcohol.

5N Aqueous Potassium Hydroxide Solution.—

Dehydroisoandrosterone Solution.—

Dissolve 25 mg. of dehydroisoandrosterone
in 100 c.c. of absolute alcohol.

This solution keeps fairly well if kept in a refrigerator.

Technic.—

Collect a 24-hour urine sample in a jug containing 15 c.c. of concentrated hydrochloric acid or 5 c.c. of 50% sulphuric acid as a preservative; or 5 c.c. of 2% thymol in acetic acid may be used, and if the specimen is kept refrigerated, not only the 17-ketosteroids, but also the compound E and compound F content can be run from the same urine.

Measure the total volume and record.

Use a 50 c.c. aliquot for the test.

Carry out hydrolysis by acidifying the sample with 10 c.c. of concentrated hydrochloric acid in a 125 c.c. Erlenmeyer flask with a ground glass stopper.

Place in a constant temperature water bath at 80° C. for 15 minutes, then cool. After cooling, the sample can be kept in a refrigerator for as long as 24 hours.

Pour the cooled sample into a 250 c.c. separatory funnel and mix twice with 20 c.c. portions of ether and once with a 10 c.c. portion, shaking 1½ minutes each time. A pinch of a detergent may aid the separation of ether layers.

Wash by combining the 3 ether extracts in a 250 c.c. separatory funnel, adding 10 c.c. portions of a freshly made sodium hydroxide-sodium hydrosulphite solution.

Wash 3 times, shaking 1 minute each time. This removes the phenolic fraction which contains estrone.

Wash the extract 3 times with distilled water, or until the washing is neutral.

Evaporate as follows:

Transfer the ether extract to a 50 c.c. Erlenmeyer flask and evaporate to dryness over a hot plate, using grooved corks. Reduced pressure for this may be obtained by using a water pump. Any remaining water can be evaporated slowly at relatively low temperatures. The cooled flask may be placed in a desiccator overnight and the dried extract can be stored indefinitely.

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[2] Holtorff, A. F., and Koch, F. C.: J. Biol. Chem. 135: 377, 1940.

[3] Callow, N. H., Callow, R. K., and Emmens, C. W.: J. Endocrinol. 2: 88, 1940.

Zimmermann Reaction:

Dissolve the dried extract in 2 c.c. of absolute alcohol.
Heat in a pan of hot water at 50° C. to aid solution, if necessary.
Use 0.2 c.c. of this extract for the reaction.

Prepare Klett tubes.

(a) **Unknown.—**
0.2 c.c. of urine extract
0.2 c.c. metadinitrobenzene solution
0.2 c.c. 5N aqueous potassium hydroxide.

(b) **Pigment Blank.—**
0.2 c.c. absolute alcohol
0.2 c.c. alcoholic extract of urine
0.2 c.c. 5N potassium hydroxide.

(c) **Standard.—**
0.2 c.c. dehydroisoandrosterone solution
0.2 c.c. metadinitrobenzene solution
0.2 c.c. 5N potassium hydroxide.

(d) **Reagent Blank.—**
0.2 c.c. absolute alcohol
0.2 c.c. metadinitrobenzene solution
0.2 c.c. 5N potassium hydroxide.

(e) **Klett Blank.—**
0.4 c.c. absolute alcohol
0.2 c.c. 5N potassium hydroxide.
Stopper all tubes and shake.

Place in the dark in a constant temperature water bath at 25° C. for 105 minutes. This causes the Zimmermann reaction, a characteristic red color which can be measured quantitatively.

Immediately add 8 c.c. of 80% alcohol to each tube, diluting in the order in which the tubes are to be read.

Mix by inversion and read on a Klett-Summerson photoelectric colorimeter within 10 minutes, using a 540 filter.

Calculation.—

$$\frac{\text{Corrected reading* of unknown}}{\text{Corrected reading* of standard}} \times \frac{\text{24-hr. volume}}{100} = \text{the 17-ketosteroids in mg. per 24 hours.}$$

Normal.—

The normal range for adults is: **males,** 10 to 20 mg., average 15 mg. per 24 hours; **females** 5 to 15 mg., average 10 mg. per 24 hours; for **children,** near 0 until the age of 8, 1.5 to 5 mg. by the age of 12 years, and then a gradual increase to adult levels at about the age of 16 years. There are no marked sex differences until this time.

There is a moderate day-to-day variation in excretion,[4] thus rendering meaningless the reporting of results to several decimal places, and diminishing the significance of "slightly high" and "slightly low" level reports.

The result of this test represents "total neutral" 17-ketosteroids, the end point for most clinical tests. Further fractionation is possible with separation into ketonic and nonketonic fractions, using Girard reagent T. The nonketonic fraction is usually 10 to 15 per cent of the total neutral results, although occasionally it may be 25 per cent. This separation does not add to the clinical usefulness of the test. However, the ketonic fraction may be further separated into alpha and beta fractions with digitonin. The beta fraction is usually under 5 per cent, but may be increased under certain conditions, and if increased to over 50 per cent, with a total excretion of over 50 mg. per day, is considered to be evidence for the presence of an adrenal cortical carcinoma. The simpler Allen[5] test is also reported to be positive in malignant adrenal tumors.

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*Corrected reading is the colorimeter reading corrected for pigment and reagent blanks.

[4] Werner, S. C.: J. Clin. Endocrinol. 1: 951, 1941.

Studies of 17-ketosteroid urinary excretion in various endocrine diseases[6] have shown low levels in hypophyseal infantilism, Simmonds' disease, Addison's disease, especially in women where excretion should be close to ''0,'' myxedema, and occasionally in anorexia nervosa. While it is true that values in Simmonds' disease are generally lower than those in anorexia nervosa, there is overlapping of the ranges in the two diseases, so that in an individual patient the test may be helpful but not decisive.

High levels are found with adrenal cortical carcinoma or malignant adenoma, and moderately high figures are found in adrenal cortical hyperplasia, especially with the adrenogenital syndrome. In carcinoma, the excretion is usually over 50 mg. per day, and may be as high as 1,000 mg. plus per day. If the excretion is over 50 mg. per day, and if further fractionation shows that over 50 per cent of this is the beta fraction, a tentative diagnosis of adrenal cortical carcinoma seems justified. In adrenal cortical hyperplasia, excretion of from normal to 100 mg. per day is seen. The Allen[5] test may help to differentiate between adrenal carcinoma and hyperplasia. Occasional instances of verified adrenal cortical carcinoma have been reported in which the 17-ketosteroid excretion has been normal. In spite of this, the test has its greatest clinical importance in this condition.

Slightly low levels are found in male eunuchoidism, male castrates, ''burned-out'' gigantism and acromegaly, diabetes mellitus, in the male climacteric, and in chronic debilitating diseases. The level tends to fall in old age.

Slightly high levels are found in Cushing's syndrome, simple hirsutism (occasionally to 25 to 30 mg. per day), active acromegaly and gigantism, arrhenoblastoma, precocious puberty, pregnancy, and in hyperthecosis.

The 17-ketosteroid excretion is affected by a number of nonspecific processes, such as inanition and debilitating illnesses. A temporary sharp rise usually follows acute trauma such as fractures, surgery, etc. Slight elevation is observed upon physical exertion.

THE VAGINAL SMEAR[7]

The vaginal mucosa of the adult woman is lined by a stratified squamous epithelium 15 to 20 cells in depth. The basal layer is composed of small well-stained cells with well-defined comparatively large nuclei. The superficial layers are lined by large flattened foamy cells with or without small pyknotic nuclei. These cells are filled with glycogen. The middle cell layers of the vaginal mucosa are composed of moderately large round to ovoid cells with a central nucleus and with varying amounts of glycogen in the cytoplasm. The thickness of the vaginal mucosa, the glycogen content of the superficial cell layers, the pH of the vaginal secretions are directly under hormonal control. When hyperestrogenism is present as during pregnancy or in conditions which may sometimes be manifest by cystic glandular hyperplasia of the endometrium, there are frequently an associated increased thickness and succulence of the vaginal mucosa, an increased glycogen content of the superficial cell layers, and a low pH (acid) of the vaginal secretions. A vaginal smear taken at this stage will show only the superficial, desquamative, large, flat cells with minute pyknotic nuclei (Plate XXXV). On the other hand, in certain types of hypoestrogenism, as in types of amenorrhea, hypopituitarism, senile vaginitis, in childhood, in the climacteric, there is a concomitant atrophy or immaturity of the vaginal mucosa. The vaginal mucosa consists of a thin layer of epithelial cells which varies in depth from one to six cells. There is failure of renewal of much of the "functionalis" cell layers, the glycogen content of the cells is minimal, and the pH of the secretions is high (alkaline). A vaginal smear taken at this stage will show few if any of the superficial desquamative cells but many small round well-stained basal cells with vesicular nuclei and many leukocytes and also occasional large oval cells from the middle layer of the vaginal mucosa (Plate XXXVI). As these basal cells mature they push upward and become larger and the nuclei become smaller. They become desquamative epithelial cells with pyknotic nuclei as they mature. Degree of maturation differs according to estrogen production or deprivation as the case may be. There are occasional exceptions to the rule for we must consider the selective action of estrogen utilization, i.e., specific organs of the body are unable to assimilate the available estrogen.

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[5] Allen, W. M., Hayward, J. J., and Pinto, A.: J. Clin. Endocrinol. **10**: 54, 1950.

[6] Escamilla, R. F.: Ann. Int. Med. **30**: 249, 1949.

[7] This section and the succeeding section were abstracted in part from Chapters 25, 53, and 54 of "Office Endocrinology," ed. 4, by Robert B. Greenblatt, M.D., published by Charles C Thomas, Springfield, Ill., 1952.

For the vaginal smear in the diagnosis of uterine cancer, see pp. 2173 ff.

The Technic and Interpretation of the Vaginal Smear

A small wooden spatula or ordinary cotton applicator is moistened with tap water. Use a speculum if necessary. Spread the folds of the vulva apart, insert the spatula into the vagina and rub gently along the lateral vaginal wall. Only the top layer, or surface epithelium should be obtained. The secretions procured are transferred to a glass slide, by passing the spatula across it. The slide is stained for 15 seconds with aqueous fuchsin and then washed. After the slide is dried, it is then ready for examination. The purpose of the vaginal smear is to determine the size and shape of the cells and nuclei; i.e., the proportion of the size of the nucleus to the size of the cell. It is well to rule out vaginitis due to monilia or trichomonads, as well as cervicitis, as this will prove of value in interpretation. Pick a clean section of the vagina, free of cervical secretion. Hence the value of using the speculum.

The interpretation of the findings in vaginal smears offers a little difficulty at first but proficiency is soon acquired. Reading of the smears varies from the negative "castrate" or 1 plus reaction, on to a positive "estrus" smear or 4 plus reaction. There are occasional inconsistencies and variations in vaginal smears from the human vagina. It is thought that in some patients there may be compensatory extraneous sources of estrogen-like substances, probably adrenal in origin. In general, the vaginal smear is a simple and reliable procedure for determining the presence of normal ovarian activity or estrogen deficiency.

Scale of the Vaginal Smear

4 Plus Reaction—large foamy desquamative epithelial cells with poor-staining cytoplasm, with or without small pyknotic nuclei; classified as positive, follicular, or estrus smear.

3 Plus Reaction—desquamative and large oval-shaped cells.

2 Plus Reaction—oval-shaped cells and occasional castrate cells.

1 Plus Reaction—basal cells, small round immature cells with large, deep-staining nuclei, and relatively small amount of cytoplasm, frequently accompanied by many polymorphonuclear leukocytes; classified as negative, estrogen deficient, or castrate smear.

Preparation of Stain

Any dye suitably diluted and filtered will suffice. Gentian violet, methylene blue, carbol fuchsin, or pinacyanole will do. Greenblatt uses a 0.5 per cent alcoholic solution of pinacyanole in 100 c.c. of absolute ethyl or methyl alcohol. The preparation will keep indefinitely. Any slides which fade may be easily restained. In the stain for simple vaginal cytology studies, vaginal smears were covered with a few drops of 0.5 per cent alcoholic solution of pinacyanole, a few drops of water were added for 10 seconds, and the slide then washed.

Physiology of the Vaginal Mucosa

Cuboidal cells line the basal membrane. Continual desquamation of the surface vaginal cells occurs. Glycogen stain of the vaginal mucosa:* superficial layers of epithelial cells are loaded with pink granular material, glycogen. An indication of the utilization of the body estrogen may be found in the amount of glycogen deposition in the vaginal mucosa. This is directly proportionate to the amount of estrogen present in the body; e.g., in pregnancy the glycogen content is very high. Among the various components into which glycogen breaks down is lactic acid which is responsible for the acid reaction of the vagina (normal pH: 4.5). Decrease in glycogen storage occurs with decrease of estrogen production; therefore if there is no glycogen present to be broken down, no acid is being produced, and the medium of the vagina shifts from acid toward alkaline. This indicates some incorrect function; and may be due either to estrogen deprivation or the presence of infection.

Vaginal Mucosa and the Vaginal Smear

1. **Newborn baby**—much glycogen is present because of mother's estrogen content. Mature vaginal epithelial smears.

2. **Child**—vaginal mucosa of several layers. Pus cells, castrate and desquamative cells in smears.

3. **Adolescence**—menses and ovulation occur. Glycogen is present, epithelial cells are large, desquamative.

4. **Postpartum period**—normal amenorrheic period with lactation from 3 to 9 months. Vaginal mucosa is atrophic. Castrate, pus cells in smears are

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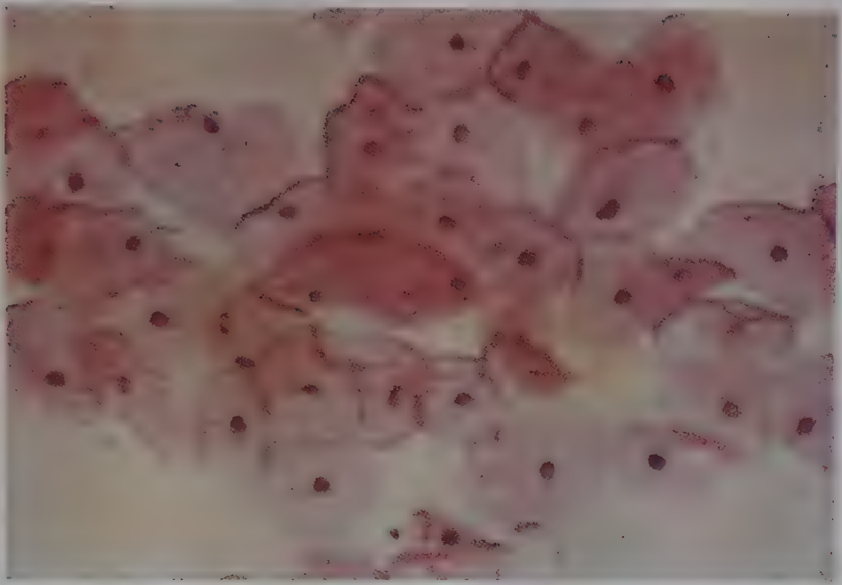
*Best's carmine stain.

PLATE XXXV.



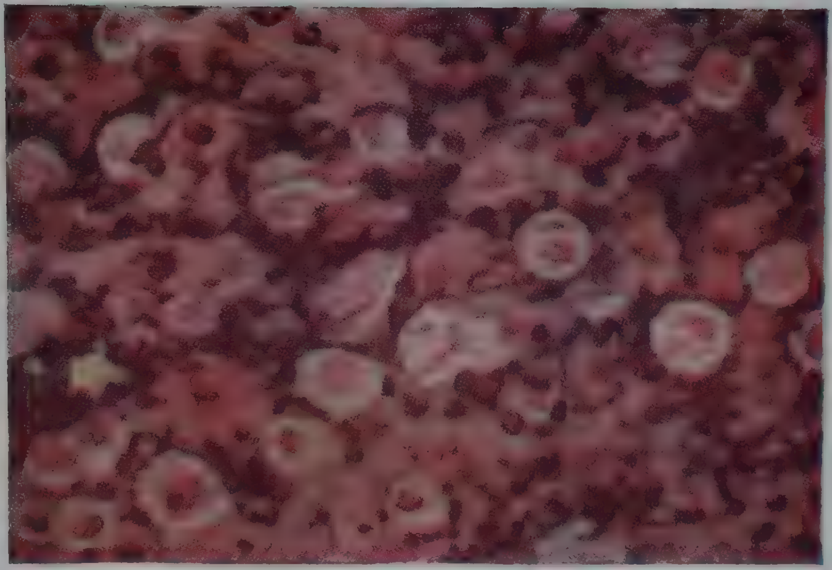


PLATE XXXVI.

PLATE XXXV.—VAGINAL SMEAR SHOWING TYPICAL 4 PLUS POSITIVE REACTION (EXCELLENT FOLLICULAR EFFECT). SLIDE STAINED WITH DILUTE CARBOL-FUCHSIN. (Courtesy Robert B. Greenblatt.)

PLATE XXXVI.—VAGINAL SMEAR SHOWING 1 PLUS REACTION. TYPICAL CASTRATE SMEAR. SLIDE STAINED WITH DILUTE CARBOL-FUCHSIN. (Courtesy Robert B. Greenblatt.)

present throughout puerperium. (Sometimes the amenorrhea continues—prolonged puerperium—this indicates that the pituitary has never become reactivated.)

5. **Menopause**—small cells, castrate, and pus cells. Glycogen deficit, atrophic vagina. (The women who have normal vaginal mucosa at this stage usually can do without estrogens).

SUCTION CURETTAGE

The Technic of Suction Curettage as an Office Procedure

Many types of curettes are in use; either the Randall curette which is smooth, or the Novak curette which possesses small teeth, is generally employed. Examine the uterus for size and position, and rule out pregnancy. Use a speculum, small or large, and clean the cervix with Merthiolate. Without touching the vaginal wall insert the sterile curette and pass it through the cervix into the uterus. The position of the curette depends on the position of the uterus; i.e., whether it is anteflexed or retroverted. Use a 10 c.c. or 50 c.c. sterile syringe with a metal adaptor or a Luer-Lok syringe. Obtain the endometrium by suction, gently scraping the surface of the endometrium and then withdrawing the curette. Wash out the curettings with normal saline. The blood and mucus are thus eliminated, and there remain only the purplish pink curettings which are then placed in 10 per cent formaldehyde and sent to the pathologist for histologic study.

The Interpretation of the Curettings as a Guide to Therapy

The study of the curettings is important for diagnoses. To interpret the curettings correctly, it is vital to understand the physiology of the menstrual cycle. The first fourteen days of the normal cycle is the estrogenic stage or the proliferative phase of the endometrium and coincides with the maturation of the follicle. When the endometrium is in the proliferative or estrogenic stage, the glands are round, uniform, and test-tube-like and are lined by a single layer of columnar epithelium which frequently has a pseudostratified appearance. The cytoplasm of the cell is deep staining and the nucleus lies at the base of the cell (Fig. 315). The supporting stroma of the endometrial glands are small and compact with deep-staining pyknotic cells resembling adenoid tissue.

From the fourteenth to the seventeenth day is the presecretory phase, which coincides with rupture and ovulation. The cells lining the glands become somewhat fuller and the nuclei descend from the base to the mid-part of the cells (Plate XXXVII). Collections of granules form at the base of the cell, and the cytoplasm becomes paler and somewhat vacuolated. Best's carmine stain now shows deposit of glycogen at the base of the cell.

From the eighteenth day to the onset of menstruation is known as the luteal or secretory phase. It coincides with the ripening of corpus luteum and the endometrium is being prepared for nidation and gestation, hence it is frequently referred to as the progestinal phase of the endometrium. The test-tube-like glands become tortuous, the nucleus moves back toward the base. The cytoplasm at the apex becomes granular, and frequently the cell membrane at the apex ruptures with expulsion of the granules into the lumen of the gland (Plate XXXVIII). Hence the secretory phase, and Best's carmine-stained sections now show the glycogen at the apex of the cell or in the lumen of the gland. Papillary projections into the lumen may be observed giving the gland a saw-tooth appearance. If the ovum is not fertilized, it dies by the twenty-fifth to twenty-sixth day; there is a sudden drop in estrogen and progesterone production and

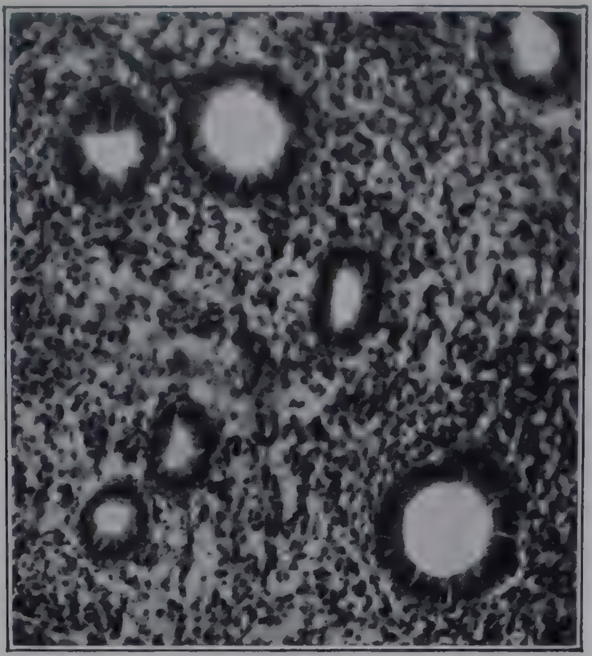


Fig. 315.

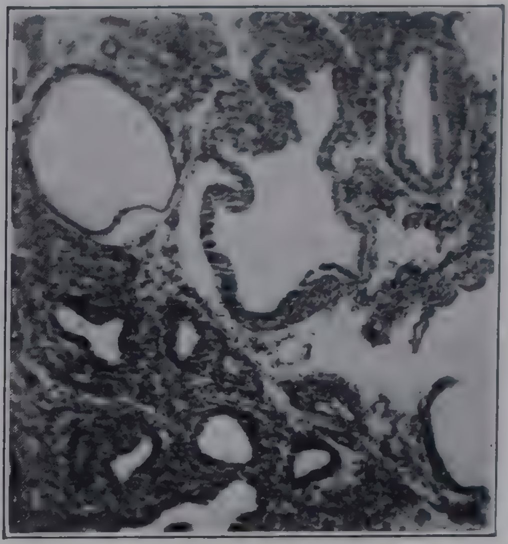


Fig. 316.

Fig. 315.—Estrogenic or proliferative phase of endometrium. (H & E stain.) (Courtesy Robert B. Greenblatt.)

Fig. 316.—Mixed or imperfect progestinal edometrium. Note that the endometrium is in various phases, i.e., some glands are secretory, others presecretory, persistent estrogenic, and cystic. (H & E stain.) (Courtesy Robert B. Greenblatt.)

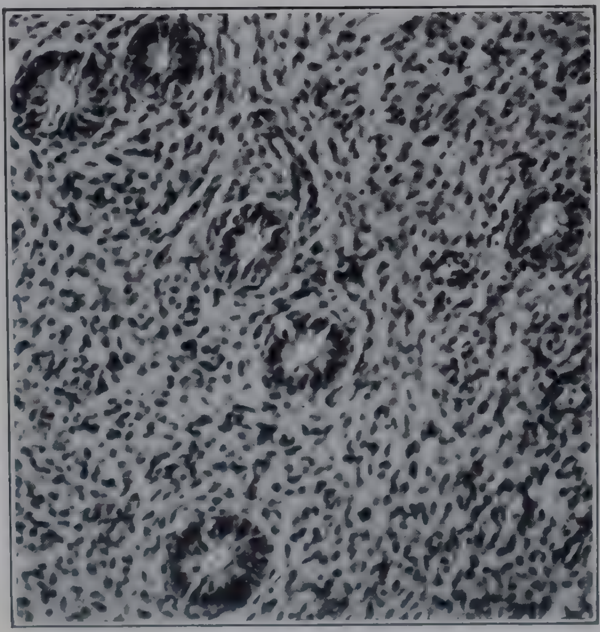


Fig. 317.

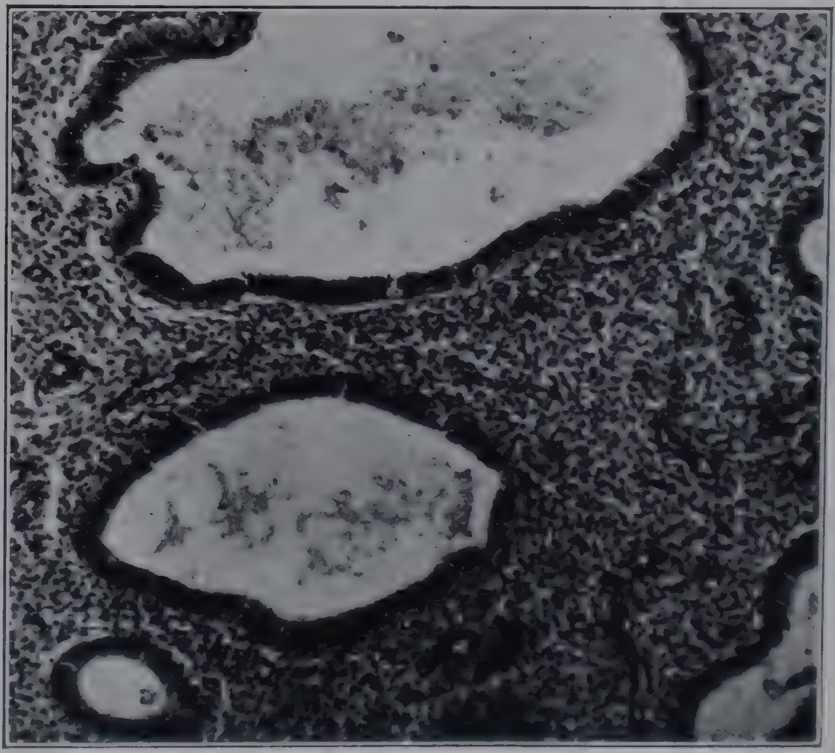
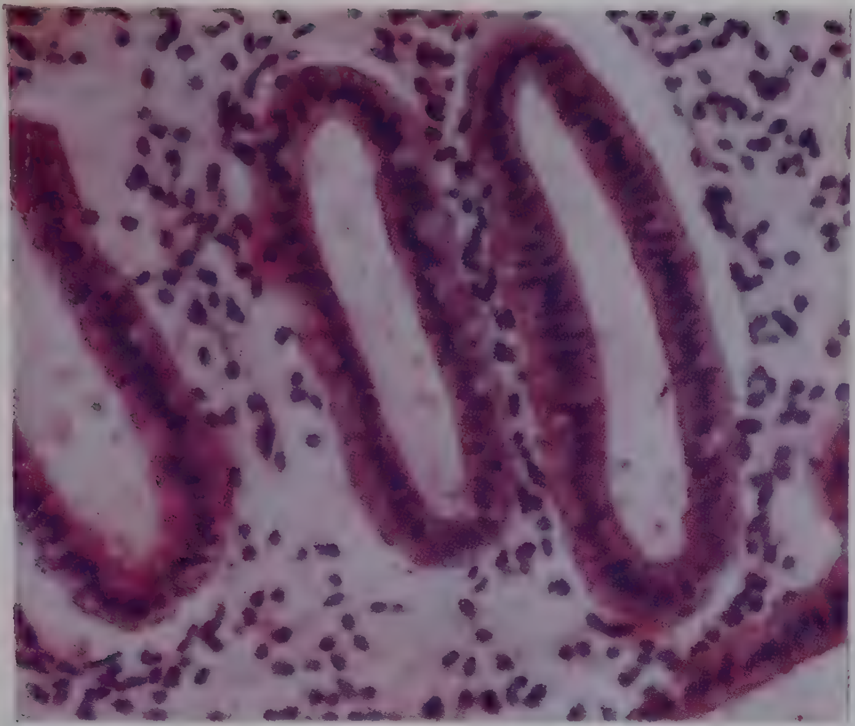


Fig. 318.

Fig. 317.—Hypoestrogenic or atrophic endometrium. Note the character of the stroma and the paucity of the glands. (H & E stain.) (Courtesy Robert B. Greenblatt.)

Fig. 318.—Cystic glandular hyperplasia or hyperestrogenic endometrium. Note the variety of sizes of the lumens of the glands. (H & E stain.) (Courtesy Robert B. Greenblatt.)

PLATE XXXVII.



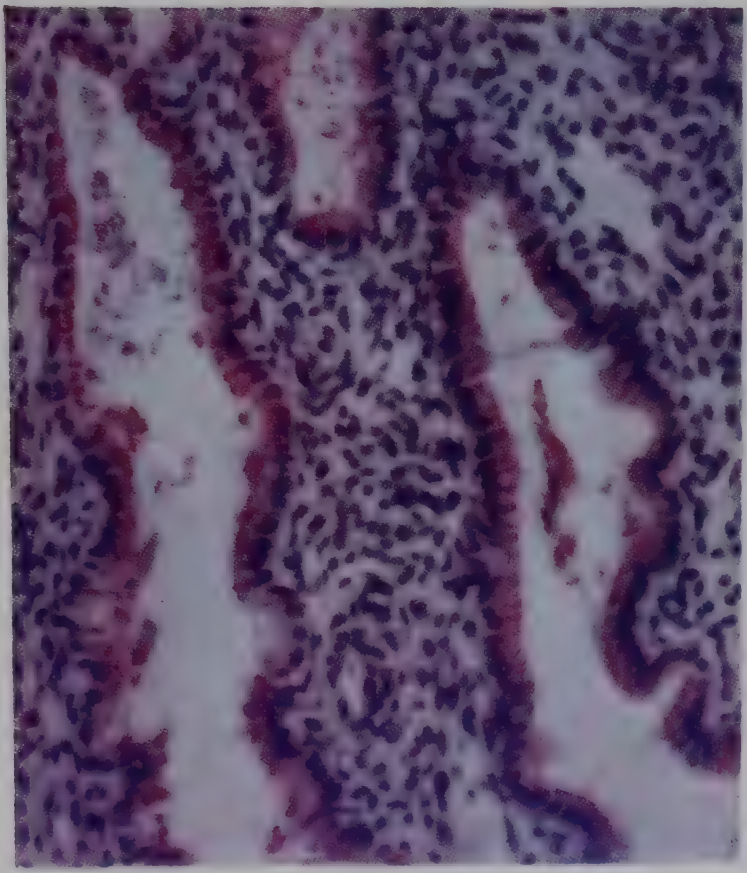


PLATE XXXVIII.

PLATE XXXVII.—PRESECRETORY ENDOMETRIUM. NOTE ASCENT OF NUCLEI TOWARD APICES OF THE CELLS. HEMATOXYLIN AND EOSIN STAIN. (Courtesy Robert B. Greenblatt.)

PLATE XXXVIII.—SECRETORY ENDOMETRIUM. NOTE TORTUOSITY OF GLANDS AND RUPTURE OF LIMITING MEMBRANE OF CELLS WITH ESCAPE OF PINK-STAINING GLYCOGEN GRANULES (SECRETIONS) INTO THE LUMEN. BEST CARMINE STAIN. (Courtesy Robert B. Greenblatt.)

menstruation occurs. Endometrium obtained at this time, therefore, is frequently referred to as premenstrual. The supporting stroma cells become loose and may take on an edematous appearance, with a vesicular nucleus and a pale spongy, eosinophilic cytoplasm. This decidua-like reaction is sometimes very striking.

In general the best time of the month in which to perform a suction curettage is on the first day of a menstrual period (especially in sterility cases in order not to disturb a possible pregnancy). The type of endometrium obtained at that time often facilitates diagnosis and is indicative of therapy; i.e., it may be of significance or value depending on whether the endometrium is follicular (Fig. 315), mixed or imperfect progestinal (Fig. 316), hypoestrogenic (Fig. 317), hyperestrogenic (cystic glandular hyperplasia) (Fig. 318), estrogenic endometrium with pseudoluteinization of stroma (Fig. 319), hyperprogestinal (progestinal endometrium with decidua-like stroma) (Fig. 320), presecretory (Plate XXXVII), or progestinal (Plate XXXVIII). Suction curettage may be performed during the last week of the cycle preferably on the first day of the menses.

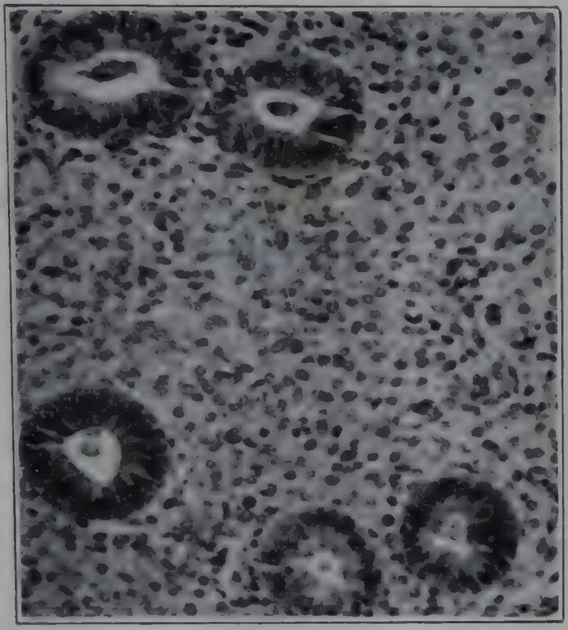


Fig. 319.

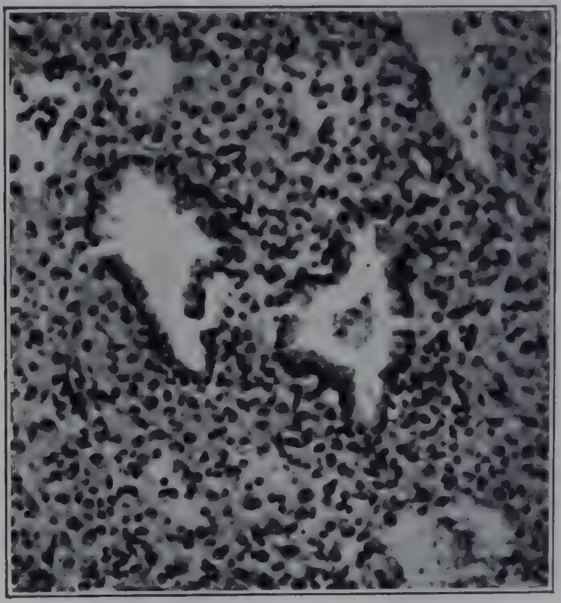


Fig. 320.

Fig. 319.—Estrogenic endometrium with pseudoluteinization of the stroma. Note the presence of decidua-like stroma. (H & E stain.) (Courtesy Robert B. Greenblatt and Edgar R. Pund.)

Fig. 320.—Hyperprogestinal endometrium. Note secretory glands and decided decidua-like stroma. (H & E stain.) (Courtesy Robert B. Greenblatt.)

The Value of Suction Curettage in Amenorrhea, Menorrhagia, Dysmenorrhea, and Other Endocrine Disorders

Amenorrhea—perform the suction curettage at least 30 to 40 days after the last menstrual period.

Menorrhagia—perform the suction curettage at any time, preferably as soon as possible after the start of a phase of bleeding.

Dysmenorrhea—perform the suction curettage as close to the onset of menses as possible.

It must be borne in mind that the endometrium per se is not responsible for the bleeding but the study of the endometrium is important for it mirrors ovarian activity. If the endometrium is atrophic, it indicates that there is an estrogen deficit which may be hypopituitary in origin or due to primary ovarian failure. If, for instance, a persistent estrogenic endometrium is obtained on the twenty-fifth day, it indicates that ovulation has not taken place, and that the proliferative phase has accordingly continued. If a cystic glandular hyperplasia is present, it indicates that there is excessive local response to the circulating blood estrogens. An imperfect progestinal or mixed type of endometrium may signify faulty corpus luteum production. A true secretory endometrium is proof of the occurrence of ovulation and corpus luteum formation.

Vaginal Smear Technic in Estrogen Treatment (Method of Papanicolaou[1])

Obtain the vaginal secretion by using a pipette made of glass tubing 8 inches long and ⅜ inches in diameter. slightly curved at one end. Attach a strong rubber bulb to produce suction, at the other end. Take the sample from the region 5 to 6 cm. within the vagina and spread on a glass slide as a heavy film, much heavier than the ordinary blood film. The new technic, based on a study of vaginal smears conducted in cooperation with Herbert F. Traut, of the Department of Gynecology of Cornell Medical College, and of New York Hospital, has advantages over the previous method described by these writers.[2, 3]

In the older methods described by these writers, the staining of the cells was too deep to permit a sharp definition of their outlines in smears that were relatively thick or contained much blood. In most cases of carcinoma and in many other pathologic conditions there is a profuse vaginal discharge frequently mixed with blood which forms a heavy film on the slides. In such rich and bloody smears there is considerable crowding and overlapping of cells which, when deeply stained, cannot be well differentiated. This applies more particularly to the small endometrial cells which are often found in menstrual and uterine bleedings and have a pathognomonic value in the diagnosis of adenocarcinomas of the fundus[2] and of other gynecologic conditions. These cells also have great importance in evaluation of the normal menstrual cycle, of sterility, and of estrogenic and other endocrine therapy.

After long experimentation it was found that a much greater transparency and an equally good color differentiation of the cells can be obtained by the use of solutions of stains in 95 per cent alcohol instead of aqueous solutions. Various alcoholic stains were thus developed; two are described below.*

Reagents.—

Fixative.—

Equal parts of 95% alcohol and ether.

80%, 70%, and 50% Alcohols.—

See page 2120.

Harris Hematoxylin.—

See page 2105.

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*For a revised method, see page 2173.

[1] Papanicolaou, G. N.: Science, April 24, 1942. For other methods of making and staining vaginal smears, see pages 2173 ff.

[2] Papanicolaou, G. N., and Traut, H. F.: Am. J. Obst. and Gynec. 42: 193, 1941.

[3] Papanicolaou, G. N.: Am. J. Anat. 52: 519, 1933.

0.5% Aqueous Hydrochloric Acid Solution.—

Add 0.5 c.c. hydrochloric acid to enough distilled water to make 100 c.c.

Orange G.—

1. *For normal slides.*
   Dissolve 0.5 gm. orange G in
   100.0 c.c. of 95% alcohol.
   Add 0.015 gm. of phosphotungstic acid (if orange G 6 is used; if orange G 8 is used, add 0.010 gm. of the acid).
2. *For cancer diagnosis.* Use 0.025 gm. of phosphotungstic acid, and orange G 5, to obtain a sharper contrast of the abnormal cell types.

Stain EA 36.—

0.5% alcoholic light green SF yellowish, N.A.	45	c.c.
0.5% alcoholic Bismarck brown, N.A.	10	c.c.
0.5% alcoholic eosin yellowish, N.A.	45	c.c.
Acid phosphotungstic, Merck	0.2	gm.
Saturated aqueous solution of lithium carbonate	1	drop

Stain EA 25.—

0.5% alcoholic light green SF yellowish, N.A.	44	c.c.
0.5% alcoholic Bismarck brown, N.A.	12	c.c.
0.5% alcoholic eosin yellowish, N.A.	44	c.c.
Acid phosphotungstic, Merck	0.17	gm.
Saturated aqueous solution of lithium carbonate	1	drop

Mounting Medium (Clarite, Canada balsam, or gum damar).

Technic.—

Make up the 0.5% alcoholic solutions first. Since the solubility of the dyes in 95% alcohol is low, heat the solutions at the time of preparation. Keep the solutions in stock without filtering. Filter stains EA 36 and EA 25 to eliminate undissolved particles of stain.

Fix smears immediately (before drying) in equal parts of alcohol and ether for 5 to 15 minutes. (Although smears may be kept in the fixative indefinitely, a prolonged fixation of a week or more affects the staining reaction of the cells.)

Rinse consecutively in 70% and 50% alcohol, then in distilled water.

Stain in hematoxylin for 5 to 10 minutes. (Staining for only 2 minutes is often sufficient, but, as a rule, better results are obtained with longer staining of 5 to 6 minutes for normal smears, and of 8 to 10 minutes for smears used for diagnostic purposes, more particularly for cancer diagnosis. For sections, even longer staining is advised. This timing applies more specifically to Harris hematoxylin, prepared with domestic hematoxylin and ammonium alum. In order to obtain more uniform staining, used hematoxylin should not be discarded, but filtered from time to time. The loss from filtering and evaporation is gradually replaced by the addition of fresh stain.)

Rinse in distilled water.

Rinse 3 to 4 times in 0.5% hydrochloric acid solution.

Rinse thoroughly in water.

Rinse in distilled water, then in 50%, 70%, 80%, and 95% alcohols.

Stain 1 minute in orange G solution.

Rinse 5 to 10 times in each of two jars containing 95% alcohol to remove excess stain.

Stain in EA 36 or EA 25 for 2 minutes.

Rinse 5 to 10 times in each of three jars containing 95% alcohol. Do not use the same alcohol which was used after the orange G.

Rinse in absolute alcohol, then xylol.

Mount in Clarite, Canada balsam, or gum damar.

Nuclei of cells are deep blue or purple; cytoplasm is light blue or blue-green; keratinized cells are various shades of red.

The advantages offered by this staining method are the following: (1) The epithelial cells and the erythrocytes are more transparent. Overlapping cells can be more easily differentiated. (2) The color of the acidophilic cells varies from red to orange. This helps in the identification of certain smear types. Basophilic cells stain green or blue-green. (3) Cells or fragments of tissue penetrated by blood take a characteristic orange or orange-green color, which permits an easier recognition of small amounts of blood, even when erythrocytes are not distinctly seen. (4) Smears which were subjected to partial or even complete drying can be stained fairly satisfactorily. The differential coloring is not entirely lost.

Short Method.—

Stains EA 36 or EA 25 can be used for short staining by those who want an immediate examination of a slide. No fixative need be used. Dip the slides directly in the staining solution or cover them by stain from a drop bottle. Thus the smears are fixed and stained simultaneously within a few minutes, although they may be kept in the stain for a longer time without being overstained. Wash off the excess stain in 95% alcohol and carry the slides through absolute alcohol, then xylol, and mount in Clarite. The nuclei are stained faintly, but the cells show good differential staining and retain their transparency. Smears stained by this simple method can be restained by a repetition of the procedure described above, including hematoxylin. This will improve the nuclear staining as well as the cellular differentiation and will permit the use of the same smears for a more detailed cytologic study.

Interpretation.—In the normal or menstruating woman, several types of smears are found at different times in the menstrual cycle. During menstruation, large numbers of erythrocytes are seen, with a few flat epithelial plates having pyknotic nuclei. With cessation of external bleeding, there is a great increase in leukocytes, and the epithelial cells are relatively scanty. At about the ninth or tenth day of the cycle, the vaginal secretion becomes much richer in mucus, and leukocytes practically disappear. About ⅓ to ½ of the epithelial cells show cornification. At the time of ovulation, a few leukocytes may appear, and the proportion of cornified to noncornified cells remains about the same. After ovulation, around the fifteenth day of the cycle, leukocytes are again very scanty, and a large proportion of the epithelial cells are cornified. As menstruation approaches, leukocytes again appear in the smear, and epithelial cells become very abundant. The latter often occur in clumps, and tend to be folded or to have scalloped edges; cornified cells are not so abundant as before. These findings may be changed by the presence of infection or parasitic infestation in the vagina, by lesions of the cervix producing discharge, etc. However, if the complete cycle of changes can be demonstrated, it is assumed that the entire mechanism of cyclical ovarian activity is functioning normally.

It is the appearance of vaginal smears made in the absence of adequate ovarian activity that is most interesting from the practical therapeutic point of view. They reflect the fact that there is pathology present. Such smears show flat epithelial cells. The nuclei, instead of being small and pyknotic, are rather large, open, and do not stain as deeply. Numerous leukocytes are present. Under treatment with estrogens, the epithelial cells become flatter, cornified, with small pyknotic deeply stained nuclei, and the leukocytes diminish in number. If one takes a portion of the mucous membrane of the vagina for biopsy, it will be found that it has resumed the appearance which is characteristic of the premenopausal years.

One must bear in mind, however, that a diagnosis must not be made from one examination and that many extraneous factors may cause irritation of the vagina, thus changing the characteristics of the smear. Just as all other laboratory procedures teach us, experience and common sense will place this procedure in its proper position as a diagnostic aid and as a measure for controlling estrogenic therapeusis.

Preparation of Gonad-Stimulating Hormone of Pregnant Mare Serum (Cartland-Nelson Method)

This is a method of fractional precipitation of the hormone from plasma using acetone or alcohol, removal of impurities by proper adjustment of acidity, and final precipitation of the hormone fraction in the presence of increased concentration of acetone or alcohol. The hormone fractions are dry, white, water-soluble powders, are stable, and may be used to prepare sterile solutions for laboratory and clinical studies.

This method was worked out by Cartland and Nelson.[1] The following chart from Cartland and Nelson gives the details of the process.

FLOW SHEET OF FRACTIONATION PROCEDURE FOR PREPARATION OF GONADOTROPIC HORMONE

16.5 liters citrated blood plasma (assay 119,000 rat units); adjust pH to 7.4 and add 16.5 liters of acetone; let stand 16 hours, separate precipitate and wash with 16.5 liters of 50% acetone

50% acetone supernatant + washings Add HCl to pH 6.0; let stand 16 hours in cold room; supercentrifuge	50% acetone ppt., pH 7.4 1,055 gm. crude protein. Assay, approximately 5,000 units (discard)
50% acetone supernatant, pH 6.0 Add acetone to make 70%, chill 16 hours, and centrifuge	50% acetone ppt., pH 6.0 45 gm. mostly protein. Assay, approximately 1,500 units (discard)
70% acetone ppt. pH 6.0 Wash with alcohol and ether and dry. Solids, 44.2 gm.; total N, 550 mg. Assay, 110,000 rat units. Redissolve in 1,000 c.c. 10% acetone and centrifuge from insoluble material	70% acetone supernatant Solids, 265 gm. salts and extractives. Assay, approximately 1,000 units (discard)
10% acetone solution Add acetone to make 50%, adjust pH to 6.0, chill 16 hours, and centrifuge	Insoluble in 10% acetone Solids, 5.38 gm. (inactive, discard)
50% acetone supernatant, pH 6.0 Dilute to 2,700 c.c. with 50% acetone, and add acetone to make 65%, chill for 16 hours, and centrifuge	50% acetone ppt., pH 6.0 Solids, 2.86 gm. Assay, 1,600 units
65% acetone ppt., pH 6.0 Wash with alcohol and ether and dry. Solids, 9.81 gm.; total N, 261 mg. Assay, 106,000 units	65% acetone supernatant Solids, 17.5 gm. Assay, 500 units (discard)

Biological Assay Method.—Follicle stimulation, ovulation, corpus luteum formation and estrus changes in the uterus and vagina may be produced in immature female rats by the injection of the mare serum hormone. Female rats of the Wistar strain colony were used by Cartland and Nelson. Weight should be 30 to 40 grams at 21 to 23 days of age. They are injected subcutaneously on each of three successive days with 1 c.c. doses of suitable dilutions of the hormone in physiologic saline. Ninety-six hours after the first injection the rats are sacrified and the ovaries dissected free of oviducts and bursae, examined for corpora lutea, and weighed. A rat unit is defined as the total dose of hormone which, administered as described above, will produce at autopsy a pair of ovaries weighing 65 mg. which is approximately five times the weight of the ovaries of the uninjected controls. A satisfactory procedure of assay is to estimate the approximate strength of a given extract by a series of preliminary injections and, on the basis of the results obtained, inject two groups of ten to twenty rats each with amounts estimated respectively to be greater and less than the 1 unit dose by approximately 25 per cent. The dose necessary to produce an ovarian weight of 65 mg. is determined by interpolation and, if desired, this may be checked by a further test.

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[1] Cartland, Geo. F., and Nelson, John W.: J. Biol. Chem. **119**: 59, 1937.

HYPERSENSITIVENESS

In the present incomplete state of knowledge concerning the phenomena of hypersensitiveness, a biologic classification is not possible. They must simply be listed by their generally recognized designations and further studied as to their mutual relationships.

Five forms are recognized: atopy, anaphylaxis, hypersensitiveness of infection (tuberculin type), contact dermatitis (dermatitis venenata), and serum disease, with which is associated drug idiosyncrasy characterized by eruption and fever and other symptoms of serum disease after an incubation period following contact with the drug. See Table 135[1] for the outstanding features of these forms.

ATOPY

Clinical forms of human hypersensitiveness not occurring in lower animals, and subject to hereditary influences, are known as atopy. Only asthma and hay fever are included, but eczema and certain forms of drug and food idiosyncrasy may later be placed with these.

Etiology.—In explanation of protein sensitization, many theories have been advanced, practically all of which are based on the belief that the susceptible person has received a previous sensitizing dose of a foreign protein. A second or provocative dose of a homologous protein, given after a certain interval, produces recognizable symptoms. It is considered necessary that both the sensitizing and the provocative dose should enter the body in an unmodified form in such a way as to be subject to parenteral digestion; for instance, by injection or inhalation. Cases of sensitization in which there is no history of a previous injection are explained by the fact that protein substances may enter the blood by way of the digestive tract unchanged, if they are present in excessive amount, or if there are gastrointestinal abnormalities. This latter consideration explains many cases of food sensitization satisfactorily, as the possibility of absorption of unchanged protein from the stomach has been demonstrated beyond doubt, especially in infants.

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[1] Coca, Walzer, and Thommen: Asthma and Hay Fever in Theory and Practice, Charles C Thomas, Springfield, Ill.

TABLE 135[1]

	OCCURRENCE	AGE AT ONSET	HOW ESTABLISHED	HEREDITY	MECHANISM	GUINEA PIG	HUMAN SKIN	DESENSITIZATION
Anaphylaxis	Occurs in lower animals, not demonstrated in man	May be acquired in utero	Induced experimentally in practically all individuals among guinea pigs, rabbits and dogs	Not especially subject to heredity	Antibodies associated with or identical with precipitin	Guinea pig uterus can be sensitized with the antibody	Cannot be sensitized with the antibody	Always possible in guinea pig and dog; difficult in rabbit
Atopy	Peculiar to man affecting 7 per cent of whites	Determined by heredity	Not induced at will	Subject strictly to heredity	Reagins not associated with precipitin	Guinea pig uterus cannot be sensitized with the reagin	Can be sensitized with the reagin	Has not been accomplished at will
Hypersensitiveness of infection	Observed in all animals susceptible to tuberculosis	Determined by infection	Results from infection	Not especially subject to heredity	Not yet found; not anaphylactic antibodies	Guinea pig uterus said to have been sensitized	Human skin cannot be sensitized passively with serum of sensitive person	Has been accomplished
Contact dermatitis Dermatitis venenata	Peculiar to man, affecting 70 per cent of whites or more	At any time of life, upon sufficient contact	Develops after contact with excitant	Not subject to atopic hereditary influence	Not yet found	**Bloch reports** positive experiments with guinea pig and rat	Human skin cannot be passively sensitized with serum of sensitive person	Has not been accomplished
"Ordinary" serum disease with possibly an ordinary form of drug idiosyncrasy	Peculiar to man, affecting 90 per cent of whites	Susceptibility present at birth	Develops during the course of contact with foreign protein	Not subject to atopic hereditary influence	Not yet demonstrated	Anaphylactic antibodies sometimes demonstrable in patients' blood	Human skin can sometimes be passively sensitized with serum of sensitive person	Not possible

[1] Coca, Walzer, and Thommen: Asthma and Hay Fever in Theory and Practice, Charles C Thomas, Springfield, Ill.

The etiological classification of hay fever, pollinosis, and allergic coryza is given by French[2] as follows:

I. Allergic

$$\text{(a) By inhalation} \left\{\begin{array}{l}\text{animal emanations}\\ \text{pollens (hay fever)}\\ \text{sachets and perfumes}\end{array}\right.$$

$$\text{(b) By ingestion} \left\{\begin{array}{l}\text{drugs}\\ \text{foods}\end{array}\right.$$

(c) By absorption from foci—bacterial

$$\textit{II. Nonallergic} \left\}\begin{array}{l}\text{infections}\\ \text{vasomoter rhinitis}\end{array}\right.$$

To animal emanations are ascribed 10 to 15 per cent of all cases of allergic coryza: 70 to 75 per cent are due to pollen hypersensitiveness. Foods and drugs and bacterial proteins are included in order to make the classification complete. It is stated, however, that no case of pure, uncomplicated coryza has ever been observed as due to food or to drugs.

''Atopens and excitants are considered under the following headings:[1]

''I. Inhalants
  A. Pollens
  B. Animal Danders
    1. Horse
    2. Cat
    3. Dog
    4. Goat
    5. Rabbit
    6. Wool
    7. Feathers
    8. Cow
    9. Hog
    10. Camel
    11. Mouse
    12. Guinea Pig
    13. Chamois
    14. Monkey
    15. Sundry
  C. House Dust
  D. Seeds
    1. Cotton seed
    2. Flaxseed
    3. Kapok Seed
  E. Miscellaneous
    1. Orris Root
    2. Glue
    3. Tobacco
    4. Pyrethrum
    5. Castor Bean
    6. Coffee Bean
    7. Insect Atopens and Excitants
    8. Mites, Fungi, Molds
    9. Various Botanical Excitants

II. Ingestants
  A. Cereals
    1. Wheat
    2. Corn
    3. Rice
    4. Rye
    5. Oats
    6. Barley
    7. Buckwheat
  B. Egg
  C. Cow's Milk
  D. Fish and Shell Food
  E. Meats
  F. Nuts
  G. Vegetables
  H. Fruits
  I. Spices
  J. Chocolate-Cocoa
  K. Honey
  L. Beverages
  M. Medicinal and Chemical Agents

III. Contactants
  A. Silkworm
  B. Cosmetics and Perfumes
  C. Poison Ivy and Primula
  D. Miscellaneous

IV. Injectants
  A. Animal Sera
  B. Drugs and Medicinal Agents
  C. Insect Bites
  D. Miscellaneous

V. Miscellaneous Excitants
  A. Parasites
  B. Physical Agents
  C. Bacteria and Their Products''

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[2] French: Clin. Med. & Surg., p. 571, Aug., 1928.

The evidence afforded by a careful study of a large series of perennial hay fever cases seems to show that the sensitivity of the patient is acquired during the second and third decades of life, or around the age of twenty to thirty years. Multiple sensitivity is the rule.

According to Coca, Walzer, and Thommen,[1] inhalants usually play little or no part in causing atopic symptoms during the first or second year of life. Food or occasionally contact offenders such as silk or wool are more often the cause of these reactions. Stuart and Farnham[2] have found the greatest percentage of food sensitivity in the second year (26 per cent of 57 positive cases in children). There is then a rapid decline, with a negligible incidence after the eighth year. Inhalant hypersensitivity, however, is infrequent during the first three years (3.5 per cent of 87 positive cases), rising rapidly thereafter until the eighth year, followed by a general decline. During the fourth and fifth years, frequencies of both types of sensitivity closely approximate each other.

Peshkin[3] also found food sensitivity most frequent in children in the second year, with a great frequency of inhalant hypersensitiveness in his series of children under fourteen years of age (98 per cent sensitive cases)—a larger incidence than food sensitivity in the same group (71 per cent of sensitive cases). Although food sensitivities occur frequently in adults, they are in a decided minority in comparison with inhalants as actual causes of asthma.

Heredity.—In certain cases of allergy there seems to be a hereditary tendency. Whole families have been found to possess an idiosyncrasy to a given protein or to several proteins, the type varying with the different members. In other instances, asthma, urticaria, hay fever, and migraine have been present in the same family. In this connection it is interesting to note that sensitization has been passed from donor to recipient in cases of blood transfusion, and also from a nursing mother to her child.

Hartwell[4] demonstrated that an excess of protein may affect the child without inconveniencing the mother. In his experiments with rats he added caseinogen in excessive quantities to the diet of the nursing mother rat. The mother remained in good health, but some of the young rats soon died, and the remainder were not successfully weaned; they suffered from convulsions and extreme exhaustion before death, a fact of great interest in view of the possible causative relationship between protein sensitization and simple or epileptic convulsions in infants and others.

Anaphylaxis[5]

Anaphylaxis is a specific sensitiveness which can be induced either actively by previous contact with the antigen or passively by contact with antibodies from another individual. Anaphylactic antibodies can be demonstrated in certain tissues, characteristically different in different species of animals; these antibodies are capable of sensitizing the unstriated muscle of guinea pig uterine strip.

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[1] Coca, Walzer, and Thommen: Asthma and Hay Fever in Theory and Practice.

[2] Stuart, H. C., and Farnham, M.: Am. J. Dis. Child. 32: 341, 1926.

[3] Peshkin, M.: Am. J. Dis. Child. 31: 763, 1926.

[4] Hartwell: Lancet, July 2, 1921.

[5] Coca, Walzer and Thommen: Ibid.

H. G. Wells[3] identifies anaphylactic sensitiveness by the following criteria:

(1) The substance injected must produce symptoms only in sensitized animals. If symptoms are produced in nonsensitized animals by injection of the same substance, anaphylaxis has not been demonstrated. (2) Symptoms must be characteristic anaphylactic reactions as observed in the usual reactions with typical soluble proteins. They must be the same for all antigens with the same test animal, but must differ characteristically with each species of animal. One must demonstrate: (3) passive sensitization by injection with sera of sensitized animals, (4) typical reactions in virgin guinea pig uterus strip, (5) by using atropine and epinephrine, amelioration or prevention of bronchial spasm in guinea pigs, (6) exclusion of possibility of capillary thrombosis or embolism when observing symptoms, (7) desensitization after recovery from anaphylactic shock.

Serum Disease

There is no generally accepted definition of this term, because of lack of unanimity as to the nature of physiologic reactions to the injections of foreign serum. The application of the term should be limited to those reactions following the first injection of foreign serum after a definite period of incubation; typically after about one week.

Frequency.—Incidence varies with different kinds of sera, from less than 3 per cent up to 6 per cent, by subcutaneous injection.

Age.—Incidence is the same throughout life, as opposed to atopic conditions (including atopic sensitiveness to horse serum) where the incidence increases from birth to middle life.

Incubation Period.—Following a first injection, the incubation period varies from three hours to longer than three weeks, with the majority of reactions occurring between the eighth and twelfth days.

Symptoms.—Fever, eruption, edema, and joint pains may all occur in one individual, but more frequently one or the other is missing. *Fever* may be as high as 106°. It may occur a day or two before the eruption, or only in the later stages. *Eruption* is usually urticarial. It may be limited to the site of the injection or may affect symmetrically all parts of the body. Local eruptions appear earlier than general eruptions. According to von Pirquet and Schick, *joint symptoms* are pain and tenderness usually without swelling. *Edema* usually affects only the face and dependent parts. Although albuminuria may accompany edema, it is never severe. *Mucous membranes* are rarely affected. Diarrhea and changes in the conjunctiva are the only changes reported in a few instances. *Lymph nodes* are enlarged, painful, and tender. Neutrophiles are sometimes lower during the attack.

Heredity.—Since about 90 per cent of the white race is susceptible to the development of serum disease, the influence of heredity does not enter into a consideration of the problem.

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[3] Wells, H. G.: The Chemical Aspects of Immunity, ed. 2, New York, 1929, Chemical Catalog Co., Inc.

Contact Dermatitis (Dermatitis Venenata)

By contact dermatitis is meant the form of cutaneous hypersensitiveness that is typified by the reactions to poison ivy in this country, and to primrose in Europe.

By atopic eczema is meant eczema occurring usually (though not exclusively) in children, excited often by some food, and subject to hereditary influences identical with or similar to that which controls the other atopic conditions of asthma and hay fever.

These two forms of cutaneous hypersensitiveness must be distinguished from each other. Atopic hereditary influence apparently controls occurrence of atopic eczema, but plays no role in the etiology of contact dermatitis. Atopic eczema frequently occurs in individuals with family history of this condition or of asthma or hay fever in later life. On the other hand, contact dermatitis may affect all or the majority of those who have been in contact with the specific excitant. In atopic eczema, application of the excitant to unaffected skin usually causes no reaction, whereas in contact dermatitis the entire skin surface is frequently susceptible to surface application of the excitant. The lesion thus produced is alike in all areas. Injecting intracutaneously specific excitant of the atopic eczema into unaffected areas of the skin usually results in wheal formation, and reagins are usually found in the blood. In contact dermatitis, however, reagins usually are not present in the blood and the intracutaneous injection of the excitant does not produce wheal formation (Cooke).

In this country, excitants of contact dermatitis are oleoresin of poison ivy and the active principle of sumac, poison oak, primrose, pollen oil, and others; certain chemicals, especially nickel salts and arsenic. These cause the condition known as ''dermatitis venenata.''

The idea that some people have a natural immunity against Rhus poisoning is erroneous. This is a typical allergy and desensitization rather than a true immunity, natural or acquired, as the case may be. Rhus poisoning is due to a single genus, *Rhus* or *Toxicodendron.* A classification adopted by the U. S. Herbarium in Washington, D. C., under the genus name *Toxicodendron,* lists the following species: (1) *Toxicodendron toxicodendron* (the former *Rhus toxicodendron or Rhus quercifolia*), (2) *Toxicodendron radicans* (eastern ivy), (3) *Toxicodendron diversilobum* (western poison oak, *Rhus lobata* or *Rhus diversiloba*), (4) *Toxicodendron vernix* (swamp sumac, *Rhus vernix, Rhus venenata*).

This subject has been reviewed by Turner.[1] He states that the toxic substance common to all these plants is urushiol. It is a dialyzable but nonvolatile oily resin. It is not air-borne, all statements to the contrary notwithstanding. Poisoning occurs by indirect as well as direct contact, and as little as 0.001 mg. will cause dermatitis. The difficulty of avoiding poisoning is increased by the fact that the plant is pleomorphic. It is thus the plant's toxicity, pleomorphism, and ubiquity which make poisoning extremely difficult to avoid by the highly susceptible. The effectiveness of two chemical herbicides, ammonium sulfamate and 2,4-dichlorophenoxyacetic acid, proved to be the first really possible solution of this public health problem. In other words, with these two herbicides, eradication of the plant in frequented areas is now possible.

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[1] Turner, C. E.: Am. J. Pub. Health 37: 7-12. 1947.

Hypersensitiveness of Infection

Hypersensitiveness of infection is a specific sensitiveness in man and lower animals that is mediated by a special mechanism.* (The word "mechanism" is used in the sense of the specific element through which the hypersensitive state is established.) In other words, the physiologic reactions of hypersensitiveness are all conceived to be caused by the interaction of a pair of specifically related substances; for example, an antigen and an antibody, or antibody-like substance, the susceptible tissues not being directly irritated by either of the two substances alone.

The term "hypersensitiveness" is used in its immunologic sense; that is, in the sense of specific sensitiveness as distinguished from its general medical sense of nonspecific hyperesthesia and also from the sense of increased reactivity.

The word "allergy" could have been used in the same sense, but this has been prevented by two circumstances. One of these has been Doerr's insistence upon a strictly etymologic interpretation of the term, making its meaning too broad; the other circumstance is the fact that in this country, at least, the word has been generally applied to the various forms of hypersensitiveness, exclusive of anaphylaxis in lower animals.

Preparation of Materials

The methods of preparing the materials and of carrying out the cutaneous tests differ according to whether the intradermal injection method or scarification method of performing the tests is to be employed.

The material generally used for the so-called "scratch" method is a powder representing the dried precipitate obtained upon treating an extract or solution of the original material with acetone. The relative effectiveness of the dry preparations and the untreated fluid preparations, which will be described presently, has been investigated in two different institutions, by Larsen, Paddock, and Alexander[1] and by Aaron Brown.[2] These observers agreed that the fluid preparations were distinctly superior to the powders, and Brown concluded that the intradermal method was superior to the scratch method when the fluid preparations were used in both.

The fluid preparations are now available commercially, but as the physician frequently finds it necessary for the diagnosis of an allergic condition to prepare a special extract, sometimes of an unusual material, the general principles which are given in making the test extracts may be followed.

The preparation of the fluid extracts must be varied according to the nature of the original material, as well as of its active substance. Since the excitants of atopic hypersensitiveness are usually insoluble in fat solvents, and, since a clear, aqueous extract is often not obtainable in the presence of fatty substances, these fatty substances are removed from the original material with some fat solvent such as ether, toluol, or carbon tetrachloride.

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*J. Allergy 1: 1, 1929.

[1] J. Immunol. 7: 81, 1922.

[2] J. Immunol. 7: 97, 1922.

For most materials, the aqueous extracting fluid is a 0.4 per cent sodium chloride, made neutral, according to the formula of Alice Evans,[3] with phosphate buffer salts.

The extracting fluid just referred to is kept in concentrated form in two solutions, 1 and 2.

Stock Buffered Saline Solutions 1 and 2

1. Sodium chloride	50.0	gm.
Monobasic potassium phosphate (KH_2PO_4)	3.63	gm.
Dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$)	14.31	gm.
Distilled water to make	1,000.0	c.c.
2. 4% carbolic acid.		

Diluted Buffered Saline

For use, add 1 part of No. 1 and 1 part of No. 2 to 8 parts of distilled water. The pH of 7.0 remains constant when sterilized by boiling. It is not suitable for pollens or house dust.

Alkaline Extracting Fluid (Coca's Fluid)

For pollens

Sodium chloride	5.0	gm.
Sodium bicarbonate	2.75	gm.
Phenol	4.0	gm.
Distilled water	1,000.0	c.c.

Pass CO_2 through the fluid until a sample of it remains colorless to phenolphthalein. Keep in tightly stoppered bottles.

Preserving Fluid

For fruit and vegetable juice

Stock:

Sodium chloride	25.0	gm.
Sodium bicarbonate	12.5	gm.
Phenol	20.0	gm.
Distilled water	1,000.0	c.c.

Diluted:

Use 1 part of the stock to 4 parts of juice for testing. Toluol must be added to prevent the growth of bacteria. If cereals are used (or flour, etc.) add the toluol to the meal before adding it to the stock. Extraction time is twenty-four hours. For danders, it is forty-eight hours, and for pollens two to three hours. Sterilize by filtration through Berkefeld or Seitz filters. If the material cannot be filtered, dialysis may be used.

Preparation of Allergens

House Dust

Store in well-covered ash cans containing an open jar of granular calcium chloride. Use the alkaline extracting fluid. Pass carbon dioxide through it for ten to fifteen minutes before using. Use enough extracting fluid to wet the material thoroughly. Add toluol to kill the bacteria. Extract for twenty-four hours. Squeeze through a press. Filter through filter paper. Dialyze for two to three days against the alkaline extracting fluid, changing the fluid twice daily, and bubble carbon dioxide through the outside fluid constantly. (Use No. 1200 cellophane sheets for dialyzing. These may be obtained from E. I. Du Pont de Nemours and Co., New York.)

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[3] Evans, Alice: J. Infect. Dis. 30: 95, 1922.

Extract first with ether to remove the fatty substances. The ether extract should be filtered to retain the small particles.

Animal Danders

1. **Horse Dander.**—Sift the curryings of cleanly kept horses through a kitchen strainer. Extract through a Buchner funnel with water-free ether until most of the coloring matter has been removed. Extract further with toluol or calcium chloride. Wash out with water-free ether. Evaporate the ether. The dander is in the ether, and remains in a cakelike mass. Break this up and remove the hairs by fine sifting. Keep the powder can dry over granular calcium chloride or in air-tight jars. This can be kept for years.

2. **Dog Dander.**—Use the clipped hair of cleanly kept dogs. Suspend in liberal quantity of water-free ether. Stir vigorously. Strain through kitchen strainers. The dander is in the ether. Proceed as for horse dander. Use 50 gm. of dog dander in 500 c.c. buffered saline. Extract for four days. Dialyze. It should contain 0.14 mg. N per c.c.

3. **Rabbit and Cat Hair.**—Epithelial scales remain attached to the hair. Extract from the ether-washed hair.

4. **Sheep's Wool** contains an extraordinarily large amount of fat. It can be extracted in water-free ether very easily by using the whole hair in the ether.

Feathers

Obtain different types of feathers from the dressers of these fowls. Feathers must not be immersed in water at any time. Stir the dry feathers in water-free ether. Treat the sediment the same as the animal danders.

Cereals

Material	Quantity	Buffered Saline	N Content in mg. per c.c.
Buckwheat	280 gm.	1,800 c.c.	2.70 mg.
Corn meal	708 gm.	600 c.c.	0.45 mg.
Orris, powdered	460 gm.	1,900 c.c.	0.18 mg.
Rice polish*	225 gm.	2,000 c.c.	0.30 mg.
Wheat flour	350 gm.	2,600 c.c.	0.75 mg.
Oatmeal	584 gm.	1,500 c.c.	0.40 mg.
Rye flour	145 gm.	400 c.c.	1.00 mg.

Extract the cereals and orris roots with ether to remove the coloring matter. Moisten the ether-treated meal well with toluol. Suspend in buffered saline twenty-four to forty-eight hours. Dialyze the oat extract. (Cereals should all be dialyzed.) Proceed as above with filtration, etc.

Seeds and Nuts

Use unroasted seeds and nuts (coffee beans, cocoa beans, etc.). Pass raw nuts and beans through meat chopper. Extract with ether frequently enough to remove the oil. Grind in a coffee grinder. Treat with ether. Grind to a powder.

Material	Quantity	Buffered Saline Extracting Fluid	N Content of Extract per c.c.
Almond	90 gm.	1,000 c.c.	3.89 mg.
Cocoanut	1 nut	1,500 c.c.	0.23 mg.
Caraway seed	50 gm.	500 c.c.	0.53 mg.
Cotton seed	40 gm.	1,000 c.c.	2.30 mg.
Mustard	1 lb.	1,000 c.c.	6.40 mg.
Green peanut	300 gm. of flour	2,500 c.c.	4.80 mg.
Green pea	420 gm. of flour	1,000 c.c.	2.90 mg.
Vanilla bean	1 lb.	1,000 c.c.	0.28 mg.
Black walnut	85 gm. of flour	600 c.c.	1.40 mg.

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*Louisiana State Rice Milling Co., 396 Greenwich St., New York.

Mustard and pepper should be treated for one week with ether to remove most of the irritating oil. Use a liberal excess and change once or twice daily. Proceed as above.

Milk

Separate the casein (casein is undesirable). Use fresh unpasteurized milk. Remove fat in a Sharples supercentrifuge at 35,000 r.p.m. Mix 800 c.c. of this milk with 2.5 c.c. of 1 per cent rennin solution. Place in a water-bath at 37° C. without stirring for one-half hour. Strain through a sterile towel. Sterilize the filtrate by filtration through a Berkefeld filter after adding 7 c.c. of saturated sodium carbonate solution. For use, dilute 1:10 and 1:100.

Egg

Dilute the egg white 1:10 and 1:100 with buffered saline solution. Sterilize with toluol over a period of three or more days. Filter sterile. Nitrogen content should be 0.32 mg. per c.c.

Meats

Treat ground-up meats with toluol to remove the fat. Let the extracts stand until precipitation is complete. Dilute with buffered saline.

Juicy Vegetables and Fruits

Press the pulp in a strong towel to obtain the juice. Four parts of juice are added to one part of preserving fluid. Let it stand under toluol six days or until precipitation is complete. Use diluted.

Poison Ivy

Use freshly collected leaves. Spread in a warm dry room on newspapers for several days until dry. Place in tin pails containing a layer of finely granulated calcium chloride over which is paper or cotton. Lids must fit tightly. Leave until dry. Prepare a coarse meal by rubbing up the leaves through a coarse meshed wire sieve. Omit the leaves and midribs. Store over calcium chloride or in stoppered jars. This preparation cannot be used after several months. Mix 10 gm. with 100 c.c. absolute alcohol. After twenty-four hours (or more), filter. It is then ready for use. (Chlorophyll can be removed with animal charcoal.)

Protein and Pollen Tests

Skin Tests for the Determination of Hypersusceptibility to Proteins and Pollens

1. Proteins.—

The proteins for the test may be made as described above, or purchased from various biological supply houses. The selection of proteins for the test is made by the laboratory. The following list is suggested:

Treatment No. 1.—Bacteria.

Catarrhalis
Diphtheroid
Micrococcus tetragenous
Streptococcus hemolyticus
Streptococcus nonhemolyticus
Streptococcus viridans
Staphylococcus albus
Staphylococcus aureus
Staphylococcus citreus
Pneumococcus Type I
Pneumococcus Type II
Pneumococcus Type III

Treatment No. 2.—Hair and Feathers.

Cattle hair
Dog hair
Guinea pig hair
Rabbit hair
Chicken feathers
Duck feathers
Goose feathers
Horse dander
Sheep wool

Treatment No. 3.—Eggs and Beverages.

Casein
Cheese
Egg whole
Egg white
Lactalbumin
Egg yolk
Cocoa
Coffee
Milk (cow)
Milk (goat)
Tea

Treatment No. 4.—Grains.

Barley
Buckwheat
Lentil
Oats
Rice
Wheat
W. Gliadin
W. Globulin
W. Glutenin
W. Leucosin
W. Proteose

Treatment No. 5.—Meat and Fowl.

Beef
Goat
Lamb
Pork
Veal
Beef serum
Horse serum
Chicken
Duck
Goose
Guinea fowl
Squab fowl
Turkey

Treatment No. 6.—Vegetables.

Asparagus
Bean
Beet
Cabbage
Carrot
Cauliflower
Cucumber
Egg plant
Garlic
Lettuce
Lima beans
Mushrooms
Mustard
Orris root

Treatment No. 7.—Vegetables and Nuts.

Parsnip
Pea
Potatoes
Spinach
Squash
Sweet potatoes
Tomatoes
Turnip
Almond
Brazil
Chestnut
English walnut
Peanut
Pecan

Treatment No. 8.—Fish.

Bluefish
Clams
Dogfish
Haddock
Crab
Halibut
Herring
Lobster
Mackerel
Oyster
Perch
Pike
Scallop
Salmon
Shad
Shrimp
Smelts
Sole

Treatment No. 9.—Fruit.

Banana
Cantaloupe
Fig
Grapefruit
Orange
Peach
Pineapple
Raspberry
Rhubarb
Strawberry

Have on hand a box of sterilized toothpicks, a bottle of 0.4% sodium hydroxide (N/10), sterile needles, gauze, alcohol, sterile water.

Give the patient a list of the proteins and instruct him to abstain from eating any of the proteins for the test to be made on the following day.

In other words, when the patient first reports for the test, make tests with treatments Nos. 1 and 2. Instruct him to eat no eggs or beverages (treatment No. 3) in the twenty-four hours' time between this day's test and the one to be made the following day. On the following day, make the tests with treatment No. 3.

Instruct him to abstain from foods on Numbers 4 and 5 for the next twenty-four hours, etc.

Do not limit the patient's food too much; for example, do not take eggs, beverages, grains, and meat from his diet in one day.

The exact order of the treatments is not important.

Method of Making the Test: Cleanse the skin of the flexor surface of the forearm with alcohol.

With a sterile needle, scratch the superficial skin, making scratches about ½ inch in length, and ½ inch apart. Make one scratch for each of the proteins in the test, and one extra scratch for the control. Do not scratch so deeply as to draw blood.

Place 1 drop of 0.4% sodium hydroxide on each scratch, including the control. Using a different toothpick for each scratch, place some of the protein extracts on their respective scratches, and rub into the skin. Do not put any protein on the control scratch. Note on which scratch each protein is placed, so as to avoid confusion in reading the results.

Allow to stand in contact with the skin for twenty to thirty minutes. Wash off with sterile water, and read.

Reading the Results: All scratches which look like the control scratch are negative. A ++++ reaction is one in which there is decided swelling around the scratch, with a wide area of inflammation.

All other degrees of reaction are judged by comparison with the ++++ and the negative reactions. Write the result of each protein after the name of the protein on the report blank.

Some laboratories prefer to inject intradermally instead of making scratches. Material for intradermal injections may be purchased from various biological houses.

2. Pollens.—

Pollens and Hay Fever.—The impression prevails among many lay and professional persons that roses, which come in the early summer, and goldenrod, which comes in the late summer, are largely responsible for hay fever. Wodehouse[1] called attention to the fact that flowers which cause hay fever are not necessarily objects of admiration, but are often associated with others, more conspicuous, which flower at the same time. The latter are thus often blamed whereas the former should be sought. When roses bloom, one usually finds nearby certain species of grass which cast out enormously more pollen than is produced by roses. At the close of summer, when goldenrod is in bloom, the less conspicuous ragweeds fill the air with infinitely more pollen from their greenish flowers.

Many plants other than grasses and ragweeds contribute to production of hay fever. Each climatic area has its own group, which flower in most localities in three distinct seasons. The *early spring* type is usually mild and of short duration. Most frequent offenders are elm, oak, poplar, birch, maple, walnut, and willow, which scatter enormous amounts of pollen over a relatively short span. This type of hay fever is therefore less important than the other two types. The second, or *early summer* type, accompanies the flowering of the grasses, which shed their pollen shortly before the trees complete their flowering, and continue until about haying time. The flowering period of these grasses is generally short, although usually longer than that of the trees, and since there are so many varieties, and their flowering periods are slightly different, the actual length of early summer type of hay fever is usually greater than that associated with the trees. Pollen irritation of grasses is more intense than that caused by tree pollens. The third period corresponds with the flowering of such plants as ragweeds, cockleburs, and goldenrods in the East, and ragweeds, false ragweeds, cockleburs, sagebrushes, and many others in the West.

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[1] Wodehouse, R. P.: Pollen Grains. Their Structure, Identification, and Significance in Science and Medicine. McGraw-Hill Book Company, Inc., New York and London, 1935, p. 134. By permission of author and publisher.

Wodehouse further states that trees which contribute their pollen to the cause of hay fever in the early spring are all wind pollinated, except the maples and willows. The former employ insects to effect pollination. The willows are both wind and insect pollinated. Birches, gray, black, and yellow, though they have flowering periods of scarcely over a week each, have been known to produce birch hay fever. Elms may be productive of hay fever. Poplars flower before the first unfolding of the leaves. Arizona cottonwood, which is a poplar, is regarded in southern Arizona and California as a frequent cause of hay fever. Pines, spruces, and cedars, with large quantities of pollen, scarcely ever cause hay fever with the exception of the mountain cedar. Walnuts of the East, black walnut and butternut, are not a serious factor, while the California black walnut is regarded as the most frequent cause of spring hay fever in the Sacramento, Napa, and Russian River valleys of California. There are many cases of oak hay fever, but in comparison with the enormous quantities of pollen which these trees shed the cases are fewer than would be expected. Sycamores are, in Wodehouse's opinion, quite harmless. In the southern states the hackberries or nettle-trees are responsible for some cases of hay fever.

The early summer hay fever is due to grasses. The low spear grass and the sweet vernal grass, which follows soon after the first flowering of low spear grass, are important contributors to this hay fever. Then comes the June grass, likewise the orchard grass, which both flower about the same time in May and continue through most of June, shedding large quantities of light, air-borne pollen. After these come redtop and timothy, flowering late in June and continuing through most of July. Bermuda grass and Johnson grass also cause hay fever in the warm and more arid regions of the United States. The late summer hay fever, Wodehouse believes, is usually due to ragweeds, the types of which are the common or dwarf ragweed of the eastern states and the giant ragweed, both flowering at almost the same time, the giant preceding the dwarf by only a few days. They are found in northeastern United States and reach almost across the continent in the northern states and Canada. They have a counterpart in the western ragweed and western giant ragweed. There are also so-called false ragweeds, of which there are probably twenty-five species in the United States. Wodehouse calls attention to the fact that the marsh elders belong to the same family as the ragweeds and false ragweeds but differ from them in that the pollen-bearing and seed-bearing flowers exist in the same heads and they lack the long spikes of pollen-bearing flowers typical of other members of the family. Therefore, they do not possess as efficient a pollen-dispersing apparatus as the others. Cockleburs are related to the ragweeds. Sagebrushes and mugworts are another group of hay fever plants, of which there are about two hundred species. They are commonest in dry regions and in greatest abundance in the Rocky Mountain states. The well-known sagebrush is an example of this weed. There are chenopods and amaranths which contain many hay fever plants.

In order to make a diagnosis of the exact species of plants which disturb hay fever sufferers, one must produce a skin reaction with an extract of the pollen which has caused the hay fever. This may be accomplished by purchasing these extracts from some well-known and reliable firm, such as Arlington Chemical Company, of Yonkers, New York, Hollister-Stier Laboratories, Wilkinsburg, Pa., or others; or one may prepare them himself if he is able to identify the pollens and prepare the proper extracts therefrom.

Methods of Collecting Pollen.—We are indebted to Dr. R. P. Wodehouse for the privilege of abstracting, from his well-known book *Pollen Grains* published by McGraw-Hill Book Company, Inc., New York and London, 1935, pages 101-105, his directions for collecting pollens in large amounts.

Special methods are needed to collect pollen in large amounts for the preparation of hay fever-pollen extracts, etc. *It is best to collect from the first flowers to open* rather than from those that bloom toward the end of the flowering season. These are more vigorous and less likely to be infested with insects. This is particularly true of the oxeye daisy and sorrel dock. The flowering period of forest trees is very short and under the influence of local weather conditions. If the weather turns suddenly warm and dry, anthesis may be completed within two or three days. These early flowering trees are pines, junipers, elms, and poplars. With these trees, the flowers reach the bursting point and remain in this condition until warm dry days come, then suddenly open and complete their pollination within one or a very few days. Collect these flowers in the cool, damp days immediately preceding anthesis.

Methods of collecting pollen vary according to whether the flowers are wind- or insect-pollinated, and whether they will continue to live and shed pollen after cutting, or whether pollen shedding ceases as soon as the flowers are cut.

Natural Shedding.—Most anemophilous and some entomophilous plants can be made to shed pollen quite naturally indoors. The anemophilous plants are the conifers and most other gymnosperms, the Gramineae, Cyperaceae, Typhaceae, Chenopodiaceae, Amaranthaceae, most of the early flowering trees, the Ambrosiaceae, and *Artemisia*. Among the entomophilous plants are some species of goldenrod, thistle, and sunflower. Cut off the flowering stems and place in water in the shedding room. Use long, shallow pans about a foot wide with a central bar arranged lengthwise to act as a catch to hold the cut ends of the stems beneath the water. Place the plants in the pans in a nearly horizontal position so that the flowering tips lean out far enough to clear the edges of the pans. Place sheets of paper under the flowers to catch the pollen. Gently tap the flowers once a day until pollen ceases to drop off, then draw out the papers and remove pollen.

Handle most of the grasses in this manner. Cut in the early morning while the dew is still on them, stripping off the leaves in the field, and trimming the stems to the proper length to fit the shedding pans. Bundle loosely, keeping the stems moist by adding water. In very dry weather, keep them immersed in buckets of water until the shedding room is reached. Do not stack the cut plants together longer than necessary, and do not bundle tightly. Respiration is rapid during this period, and generation of so much heat and moisture may "burn" the plants and completely destroy them in less than an hour.

Since many grasses exhibit a marked periodicity throughout the day in their shedding, collect all of them in the morning. Place in the shedding pans as quickly as possible, to prevent drying and consequent acceleration of the opening of the anthers with shedding of the bulk of the day's pollen.

Some grasses, like Indian rice and tall oat grass, shed little or no pollen after being cut. Place them in shedding pans for two or three days, during which time most of the anthers will have dropped off unopened. The rest can be removed and the flowers gently shaken. Brush up the anthers, partially dry, and shake on sieves to remove any pollen that can be freed. Then pass the anthers through a coffee mill with plates adjusted just tight enough to roll them and loosen the pollen. If properly handled and the anthers are not too dry and brittle, most of the pollen can be separated by sifting. The proportion of immature, anaphylactogenically less active grains obtained by this method depends upon the skill and care with which the grinding and sifting are done.

Plant-Breeder's Method.—(For plants which do not shed naturally indoors.) Tie paper bags over the flowers in the field just as they begin to pollinate. Bags must be moisture-proof, but porous enough to permit of escape of moisture from within. Leave on only a few hours, then remove, empty the pollen, and dry it.

Most insect-pollinated flowers are more difficult to handle. Use the following method for roses. Gather the flower buds just as they are about to open. Dry slowly on sheets of paper in a warm, dry, well-ventilated room. Pull off the tightly closed corollas from their receptacles, and empty the pollen on to a sieve. Brush through to separate it from the anthers and other floral parts.

Carbon-Tetrachloride Method.—(For entomophilous plants.) (Wodehouse, 1916.) Dry but do not completely desiccate the staminate flowers. Remove from their floral receptacle and free as far as possible from all extraneous material. Soak the mass of flowers in carbon tetrachloride in an open pan or mortar, beat gently with a pestle to loosen pollen, then empty onto a piece of fine muslin stretched across another pan, and press out the carbon tetrachloride, carrying the pollen through the muslin. Wash the crushed flowers repeatedly with carbon tetrachloride to loosen and suspend all the pollen. Pour onto a Büchner funnel fitted with smooth filter paper, and draw off the carbon tetrachloride rapidly by suction. The pollen collects on the filter paper in a solid cake. Wash with fresh carbon tetrachloride to remove lipoids, and pass an air current through to dry it. Break up the cake, sift the pollen through a 200 mesh sieve, and desiccate. Contaminations are usually negligible.

Desiccation.—Never omit complete and prolonged desiccation, since moisture in the pollen will cause it to cake and become useless; desiccation prevents deterioration which air-dried pollens undergo. As soon as possible, brush up the pollen, sift, and dry in a vacuum desiccator over calcium chloride or sulphuric acid until all moisture is removed, then transfer to airtight containers with as little exposure to air as possible. Even desiccated pollen may take up moisture so rapidly that it is difficult to weigh it in ordinary atmosphere.

Preparation of Pollen for Microscopic Examination.—Great interest has been evoked by the study of hay fever, its diagnosis by skin reaction, treatment, etc. The following material is condensed from Wodehouse, *Pollen Grains.**

Methyl-Green Glycerin Jelly.—Pick up a small amount of pollen on the flat end of a toothpick, place on the center of a glass slide, and add a drop of alcohol. Allow to evaporate partly and add a second and third or even fourth drop if necessary. Wipe off the oily ring which is deposited around the specimen, using cotton moistened with alcohol. Before the specimen is completely dry, add a small drop of hot melted methyl-green glycerin, stir the pollen in with a needle and distribute evenly. Keep the jelly hot by passing the slide over a small flame, using just enough heat to sting but not burn the knuckle. Pass a No. 0 cover glass several times through the flame, holding vertically with forceps, then place over the specimen, and gently heat. The cover should settle into position, the gelatin reaching the periphery just when the pressure of the cover begins to be taken up by the pollen grains. The amount of jelly must be learned by experience. Small amounts leave the grains crushed or flattened or the mount incompletely filled, while large amounts cause too thick a preparation for oil immersion lenses.

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*Wodehouse: Pollen Grains, McGraw-Hill Book Company, Inc., 1935, pages 106-109.

Naturally-shed pollen is preferable, although satisfactory results are obtained from herbarium specimens if they were quickly and completely dried. Pollen may usually be obtained by tapping the dry flowers over the slide or crushing a few anthers on it. If this is unsuccessful, remove a few anthers or, with the Compositae, a few florets from the specimen, and place on slide. Moisten with alcohol, add a drop of water, and heat to boiling. Tease out the pollen, remove anthers and debris, leaving pollen in the water. Draw off the water with cotton or filter paper and add glycerin jelly.

Glycerin Jelly (Brandt[1]).—Soak gelatin 2 or 3 hours in cold water, pour off excess water, and melt by heat. To 1 part of this add 1½ parts of glycerin, and while still hot, filter through spun glass pressed into the lower part of a heated funnel. Add 2 or 3 per cent phenol. Keeping mixture hot and fluid, add drop by drop a saturated solution of methyl green in 50 per cent alcohol, until the glycerin jelly becomes as dark as green ink. Or add the dye to a good commercial preparation of glycerin jelly. Pollen grains rarely become over-expanded in this medium because the relative proportions of glycerin and water are properly balanced.

Contrast Stain.—Only the exine stains with the above, leaving the intine and cell contents unstained. To obtain a contrast between exine and cell contents, stain pollen first by placing on slide in a weak aqueous eosin solution.

Pollens mounted in methyl-green glycerin jelly are not permanent preparations. They show greater brilliancy for the first few days after mounting, retaining their brilliancy for several months. The dye fades out in from about 9 months to 2 years, after which they are completely bleached. They may be restored by heating the slide to remove the cover glass, and working in a small drop of hot methyl-green glycerin jelly with a needle, and adding a fresh cover glass.

Aqueous fuchsin is just as selective as methyl-green and is *permanent.* Fischer's (1890) method follows: After washing the pollen on the slide with alcohol, add a drop of weak basic fuchsin solution in water, and stir pollen with needle. When the grains have taken up all the dye, draw off water with cotton. Do not allow to dry. Add a drop of melted glycerin jelly, stir in the pollen, keeping the slide warm. Cover with No. 0 cover glass.*

Atmospheric Pollen Slides.—Place a small drop of melted glycerin jelly containing either methyl-green or fuchsin on a slide, spread out to occupy an area equal to and of the same shape as the cover glass which is to be used, expose in a horizontal position protected from rain and sun by a shelter at least 4 inches above it. After 24 or 48 hours, examine in the laboratory with a hand lens, and remove extraneous material like soot or sand. Heat the slide, controlling temperature with knuckle to drive off excess moisture. Cover with No. 0 cover glass.

To make a pollen count, place the slide on the microscope, use mechanical stage, pass from left to right and back again progressing the width of a single field in a forward direction with each passage from side to side. Use low power. Identify pollen grains if necessary with high power. Continue counting until the entire surface has been covered.

Examination of Dry Pollen.—Shapes of unexpanded pollen grains must be observed dry in air with or without a cover glass. These observations are useful in seeing how much oil naturally occurs on the surface of the grains and as a check against the method described below. Shapes of the grains can be learned with this method and something of the mechanical action of their organs of volume-change accommodation. For a detailed examination, stain, and bring into a medium of suitable refractive index as follows:

Aniline-Oil Gentian Violet Method.—Place pollen on slide, add two or three drops of aniline oil tinted with gentian violet to a pale purple color. Heat gently over a small flame, controlling temperature with knuckle. Heat until grains are deeply stained. Cool to room temperature. Draw off excess oil with filter paper. Wash by repeatedly adding xylol and drawing it off until all oil and unabsorbed dye have been removed. Mount under Canada balsam. Grains are unexpanded, but are brilliantly stained and in a medium suitable for oil immersion observation.

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*A very satisfactory glycerin jelly for this purpose is prepared by Elmer and Amend, New York.

[1] Lee: Microtomist's Vade Mecum, ed. 7, p. 242.

Skin Tests to Determine Sensitization to Pollens

The following list of pollens is suggested:

Dandelion
Sweet white clover
Daisy
June grass
Sunflower
Sweet vernal grass
Ragweed short
Ragweed giant
Timothy
Alfalfa
Rose
Orchard grass
Corn
Red top
Goldenrod
Sweet clover
Golden glow
Cockleburr

The test is performed like the protein test, except that the 0.4% sodium hydroxide is omitted, since the pollens are in a liquid state. The control scratch, however, has a drop of sodium hydroxide solution on it. Read as in the protein tests.

Cytology of Allergic Secretions From the Nose and Paranasal Sinuses*

The shock organs or tissues of the body are those in which the lesions of allergy occur. In these shock organs the pathologic picture is characterized by edema and eosinophilic infiltration. In the nasal and paranasal sinuses, as well as all other organs which are termed shock organs, bronchial, conjunctival, salivary gland, pancreatic, intestinal, and genitourinary systems, these eosinophiles migrate through the epithelium and are easily found in the secretions of these organs. This reaction of the shock organs can be demonstrated by an examination of the secretions for eosinophiles. Warthin Jones[1] in 1846 first recorded this observation. Since then, many others have repeatedly called attention to it, namely, Ehrlich[2] in 1879, Bizzozero[3] in 1887, Gollasch[4] in 1889, Schmidt[5] in 1891, Lewy[6] in 1891, and Neusser[7] in 1892.

It is accepted that suppurative infections of the upper air passages can be differentiated from nonsuppurative infections by means of the smear examinations of the secretions. Many of the nonsuppurative, hyperplastic, or polypoid diseases of the nose are of allergic origin. The cytologic examination of secretions from nasal sinuses is always a biopsy. This is a very important contribution to the diagnosis of allergic conditions because many cases of hemorrhage and gastrointestinal disease of allergic origin may be confirmed by cytologic examinations of the nasal secretions.

Method of Collecting the Specimen.—Specimens should be taken separately from each side of the nose. Secretion is easily collected by having the patient blow his nose and collect the secretions on a piece of wax paper or cellophane handkerchief. This specimen represents a collection of both nasal and sinus secretions. If no secretion is secured by blowing the nose, it may be necessary to obtain the specimen by swabbing with a cotton applicator. The insertion of a saline tampon into the nose may stimulate the flow of secretion.

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*This section is abstracted from a report by Hansel, French K.: J. Allergy 5: 357, 1934.

[1] Jones, Warthin: Philosophical Trans. London, 1846, p. 63.

[2] Ehrlich: Verhandlung der physiologischen Gesellschaft zu Berlin, 1879.

[3] Bizzozero, G.: Handbuch der klin. Mikroscopie, Erlangen, 1887.

[4] Gollasch: Fortschr. d. Med. 7: 361, 1889.

[5] Schmidt, A. D.: Zentralbl. f. in klin. Med. 12: 373, 1891.

[6] Lewy, B.: Berl. klin. Wchnschr. 34: 845, 1891.

[7] Neusser, E.: Wien. klin. Wchnschr. 5: 41, 1892.

A number of conditions determine variations; such as, the quantity and quality of the secretion available for examination. During the periods of reactions the secretion may be thin and watery with very little, if any, mucus present. This material may show eosinophiles only after many examinations; often they are found clumped in small masses or clusters. If mucoid material is obtained, an abundance of eosinophiles may be found. If the mucous membrane itself is examined, the maximum infiltration of the eosinophiles is noted just beneath the basement membrane. They migrate from the basement membrane to the surface where they are uniformly distributed. They are then collected in the mucous secretion which is rolled or propelled toward the nasopharynx by ciliary action. Very profuse mucoid secretion is attended with very few cells. Immediately after a period of reaction and during the quiescent stage following, an abundance of thick mucus may be found collected in the nasal cavity; this secretion usually contains an abundance of eosinophiles.

The color or consistency of the secretion is not an index of the cytologic contents. Clear secretion may contain many eosinophiles or many neutrophiles or it may contain varying proportions of both types of cells. Yellowish secretion contains a marked predominance of eosinophiles.

Staining Technic

Method No. I

The method given below was developed by Hansel.[1] Hansel found that, because of variations in the pH, minerals, and viscosity of secretions from the nose and paranasal sinuses, Wright and Giemsa stains are often very inconsistent.

Allow smears to dry in the air, or dry gently over a flame.

Mark across the slide next to the label with a paraffin stick to prevent overflow of stain.

Cover completely with Hansel stain* and allow to stand for 30 to 45 seconds, giving the longer period to thicker or milky smears.

Add distilled water to take up the stain, as in the Wright stain technic, and allow to stand 30 seconds.

Pour off the stain and flood the slide with distilled water to remove the excess stain.

Flood the slide with 95% ethyl or methyl alcohol.

Drain off and dry the slide over a flame.

Wipe the back and edges of the slide with a piece of wet paper towel to remove excess stain.

Caution: If the alcohol is left on too long, or used to excess, it will wash the blue stain out of the neutrophiles and cause them to appear pink. Pink neutrophiles present a clear homogeneous cytoplasm. There are no true granules and they should be readily distinguishable from the eosinophile, which stains definitely red with deep red granules and a prominent blue nucleus. Free red granules often appear from broken eosinophiles.

Mount the slide in gum damar or Canada balsam.

Stains should be kept in amber bottles, of the dropping variety. The stopper should be turned when not in use to prevent evaporation.

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*Made by the Lide Laboratories, Inc., 634 N. Grand Blvd., St. Louis 3, Mo.

[1] Hansel, F. K.: Clinical Allergy, St. Louis, 1953, The C. V. Mosby Company, p. 411.

Method No. II

The method is essentially the same as that used for blood films. Smears must be absolutely dry before staining. Fix 1 minute in methyl alcohol after drying. Use Giemsa stain as the stain of choice. Eosinophile granulations are brownish red with a blue nucleus. Neutrophiles show a clear violet cytoplasm with a deep blue nucleus. Giemsa stain is diluted in the same manner as for blood films; namely, one drop of stain is added to each c.c. of neutral distilled water. About 1.0 c.c. or 1.5 c.c. of stain are needed for each slide which should be fully flooded. Allow the stain to remain in contact with the slide for thirty minutes. Pour off and wash gently in neutral distilled water. A few drops of ethyl alcohol should be poured over the slide to clear excess stain. Stand the slide on end to dry, mount in gum damar or Canada balsam, and then examine under low-, then high-power magnification. Thick grossly visible areas on the slide may be examined first with the low power and then with the high power. Note the character of eosinophiles. If stained too long or too heavily, they may appear quite dark. Do not mistake neutrophiles for eosinophiles. Neutrophiles contained in dense masses of mucus may show false staining, probably due to degeneration. They may take the eosin stain in excess and appear quite like eosinophiles, but upon close examination it will be noted that true eosinophilic granules are not present. The cytoplasm stains very faintly, but the nuclei stain well. The margins of thick collections of cells stain very well.

In the interpretation of the cytology of the secretions one must take into consideration the type and quantity of secretion in the specimen and correlate the findings with the clinical history and nasal changes. The clinical type of allergy under consideration and the possible relationship of acute and chronic infection must always be evaluated. An appreciation of the significance of the cytology of secretions can be accomplished only by individual experience and then only after the examination of many hundreds of specimens. If one has this work done by a laboratory or by a technician the clinician sacrifices the real value of cytologic findings, for individual interpretation must be correlated with all the clinical facts.

In the inspection of a smear, in one containing only eosinophiles, for instance, it will be noted that the cells are irregularly distributed. Sometimes they are scattered, arranged in clumps or arranged in streaks. (Plate XXXIX, A, B.) When mixed with neutrophiles they may likewise be irregularly distributed. Only certain parts of the slide may show significant areas. It is not unusual, for instance, to find a slide completely covered with neutrophiles except in certain small areas where eosinophiles are clumped in masses.

In recording the cytologic findings the question arises as to what percentage of eosinophiles constitutes a diagnostic number. In view of the irregularity of distribution, it is quite impossible to make an accurate evaluation in terms of percentages. One can learn only by individual experience how to evaluate the cytology. In making an accurate record of the cytologic findings on a history the only method is a colored photograph, but since this is not practical, the next best method is the use of the terminology employed in recording the results of skin tests. The plan was adopted, therefore, of recording the cytologic findings of eosinophiles and neutrophiles in terms of ±, 1+, 2+, 3+, and 4+. Although many epithelial cells may be noted in specimens, we have attached no definite significance to them. In using this plan of recording the cytology, due consideration must be given to the type of specimen under examination. A scanty amount of secretion may show a small

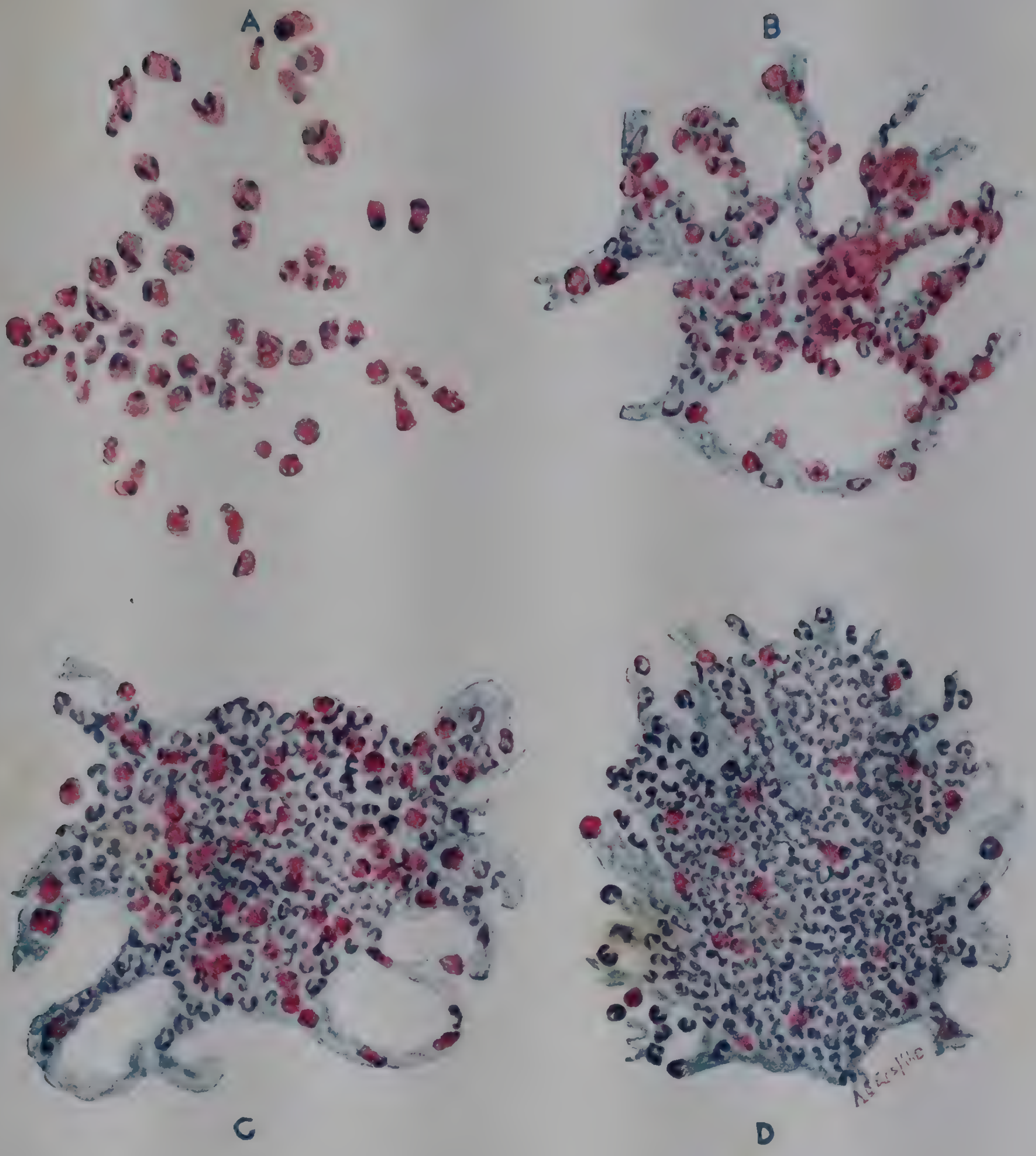

Plate XXXIX.—Nasal Secretion. Giemsa Stain, ×400. Four Representative Fields Showing Eosinophiles and Neutrophiles. All Diagnostic of Allergy.

A. Eosinophiles arranged in a cluster. These clusters are frequently found in thin watery secretion but may assume this arrangement in association with neutrophiles.

B. Eosinophiles with a few scattered neutrophiles, enmeshed in streaks of mucus. This arrangement is most frequently observed.

C. Eosinophiles and neutrophiles, with a slight predominance of the latter. This picture is observed in the resolution stage of acute infection or in chronic infection; commonly noted in polyp cases.

D. Marked predominance of neutrophiles. Approximate diagnostic minimums of eosinophiles. Usually seen in complicating acute infection, but may be noted in chronic infection. This number scattered over several fields often observed in the resolution stage of acute infectious rhinitis in nonallergic patients, especially children.

(From Hansel: Journal of Allergy, May, 1934.)

TABLE 136.—INTERPRETATION OF CYTOLOGIC PICTURES*

<table>
<tr><td colspan="3">E+ E++ E+++ E++++
Pure allergic cytologic pictures with no complicating infection. Number of eosinophiles varies according to cell dilution or concentration in thin, moderately thick secretion, or according to clumps.</td></tr>
<tr><td colspan="3">N+− N+ N++ N+++ N++++
Normal or infectious cytologic pictures with no eosinophiles. N+− and N+ normal or quiescent stage. N++ may be onset or end stage of a cold. N+++ and N++++ acute, subacute, or chronic infection. May be noted in allergic as well as in normals.</td></tr>
<tr><td>E+− N+
E+ N+
E++ N+
E+++ N+
E++++ N+
Allergic pictures with mild secondary infection, usually caused by obstruction of nasal respiration. E+− indicates few scattered eosinophiles. No clumps. Not diagnostic of allergy.</td><td>E+− N++
E+ N++
E++ N++
E+++ N++
E++++ N++
Allergic pictures with secondary infection. Stagnation caused by marked nasal obstruction. May be onset or end stage of an infection.</td><td>E+− N+++ E+− N++++
E+ N+++ E+ N++++
E++ N+++ E++ N++++
Marked infection. E+−, N+++ not diagnostic of allergy, may be observed in normals as well as allergics. E+, N+++, allergic individual with an infection. E++, N+++, E++, N++++, onset or end stage of acute infection in allergic individual, or superimposed chronic infection. Eosinophiles usually clumped.</td></tr>
</table>

E=Eosinophiles. N=Neutrophiles.

*Hansel, F. K.: Clinical Allergy, St. Louis, 1953, The C. V. Mosby Co. By permission of the author and the publisher.

but significant number of eosinophiles, while a good specimen may show a four-plus number. Table 136 and Plate XXXIX show the method of recording the cytologic findings in terms of eosinophiles and neutrophiles.

Mild reactions occur in the mucous membrane without definite symptoms, as seen by the presence of large numbers of eosinophiles during quiescent periods. Eosinophiles may occur in nasal secretions in patients with almost symptom-free seasonal and nonseasonal allergy and with symptom-free asthma, although during quiescent periods between exacerbations, most patients show no typical changes in the nasal mucous membrane.

When both eosinophiles and neutrophiles appear in the same secretion, a superimposed acute or chronic infection is indicated. Neutrophiles usually outnumber eosinophiles about ten to one. Thus a one-plus or plus-minus neutrophilia represents about ten times as many eosinophiles. Four-plus neutrophiles means the field is completely covered with these cells.

Neutrophiles are found in normal noses in winter when infections are prevalent and environmental influences must be considered. Obstruction and dryness cause a few neutrophiles at any time during the year. Neutrophiles are found more frequently in older patients. Marked obstruction in the nose in severe reactions causes retention of secretions with consequent stagnation and possibly secondary infection with resultant neutrophilic response. In seasonal hay fever with marked obstruction, a number of neutrophiles may be present. With hyperplasia of the membrane with many ciliated epithelia replaced by nonciliated types, secretions may remain in the nose for long periods and secondary infections may develop. Nasal polyps produce the greatest degree of stagnation and secondary infection, although in many of these cases eosinophiles may be found in the nasal secretions in preponderant numbers. Cytology of secretions in such cases may be the deciding factor in determining presence or absence of complicating acute or chronic infection. If acute infection is present, make repeated smears over a period of several weeks before deciding definitely as to the existence of a chronic type of infection. Persistence of marked predominance of neutrophiles indicates chronic infection. During the resolution stage of acute colds in nonallergic patients, eosinophiles are found mixed with neutrophiles in the secretions, the number sometimes approaching the number found in allergic patients during acute stages of infection. Repeated follow-up smears must be made to differentiate between the two types. Eosinophiles disappear before the neutrophiles in nonallergic cases, while in allergy the eosinophiles increase as the neutrophiles disappear. Finally a pure eosinophilic response is present. Chronic infections may obscure the picture. An acute exacerbation of allergy and an acute cold may be so similar that in many instances it is quite impossible to differentiate them on the basis of clinical history.

A pure eosinophilic response is indicative of a pure allergic process. A few eosinophiles and no neutrophiles is a very significant finding and indicates that a scanty amount of material has been obtained. A large number often clumped in large masses can be found. The eosinophilic-neutrophilic cellular collections noted in smears represent varying degrees of superimposed infection. Secretion with a few scattered eosinophiles and neutrophiles (E±, N±, 1+) is highly suggestive of allergy and should be checked by further smears. The slight neutrophilic response should be correlated with the clinical history and the nasal changes. The presence of a few eosinophiles and numerous neutrophiles (E±, N 2 to 4+) indicates superimposed infection usually of the acute type. (Plate XXXIX, D.) During acute infection the eosinophilic response in allergy is markedly decreased or almost absent during the first few days of the process. The presence of many eosinophiles with a comparatively small number of neutrophiles (E 1+, N± or 1+) indicates the existence of definite allergy with mild infection, usually not of great significance. This may indicate anatomic abnormality or the end-stage of an acute infection. Secretion with many eosinophiles and an abundance of neutrophiles represents a definite allergic process with superimposed acute or chronic infection. Only repeated observations may determine which type of infection is present. A large number of eosinophiles with a comparatively small number of neutrophiles indicates an active allergy with mild infection, usually of no particular significance (E 2 to 4+, N± to 1+) (Plate XXXIX, B). Large numbers of eosinophiles and neutrophiles (E 2 to 4+, N 2 to 4+) may represent the resolution stage of an acute infection or a chronic infection. (Plate XXXIX, C.) This cellular response is frequently found in cases of nasal polyps.

With atypical symptoms and no characteristic changes in the nose, or with thickening or polypoid changes, where a possibility of allergy exists, cytology of the secretions must be relied upon to establish a diagnosis definitely. In infection, with no eosinophiles, allow the infectious process to subside before making a diagnosis.

Hansel* summarizes the question of demonstration of eosinophiles as proof of allergy in nasal secretions as follows:

1. Eosinophiles in secretions from the nose and paranasal sinuses are good presumptive evidence of allergy.
2. Neutrophiles present with eosinophiles indicate superimposed infection.
3. Proportion of eosinophiles and neutrophiles is an index of the nature of the infection.
4. Acute and chronic infections can be differentiated by repeated observations of the cytology of the secretions.
5. Clinical data do not substantiate pure eosinophilic response as a result of bacterial hypersensitiveness.
6. Hyperplasia and polyposis with persistent absence of diagnostic number of eosinophiles are usually not allergic.

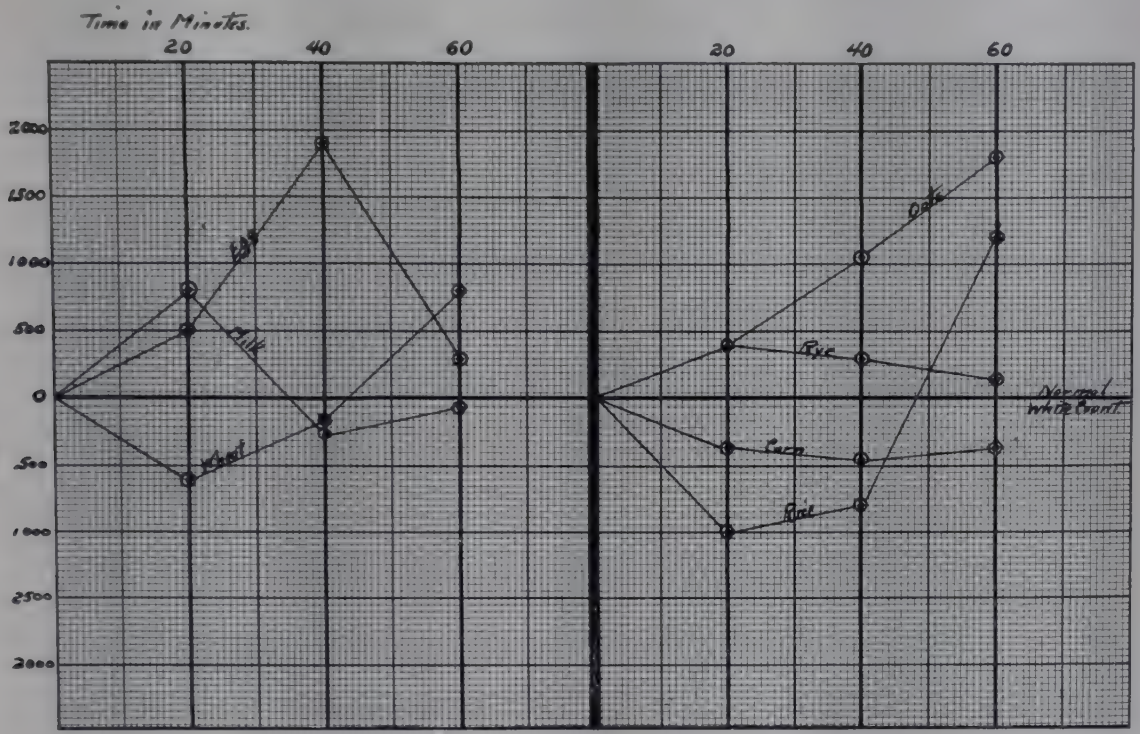

Fig. 321.—Leukocyte response to various food products. In both graphs, the curves which extend below the normal line are indeterminate, or borderline; the other curves show a normal reaction to ingestion of food. Thus milk, wheat, corn, and rice are indeterminate, while the other curves are normal. (Courtesy French K. Hansel, St. Louis.)

The Leukopenic Index

Hansel[1] states that the term *leukopenic index* was originally suggested by Vaughan[2] to apply to a series of total leukocyte counts performed as a diagnostic measure in food allergy. Normal individuals usually show a leukocytosis of 1,000 to 2,000 or more cells within 30 to 60 minutes following the ingestion of food. Widal[3] observed that after the administration of food to individuals sensitive to these food proteins there was a drop in the leukocyte count within 20 to 60 minutes after the ingestion of this food. Vaughan noted that if an individual is allergic to a food, there is usually a leukopenia of 1,000 or more cells following its ingestion.

Using the above information, the leukopenic index test was devised.

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*Loc. cit.

[1] Hansel, French K.: Lab. Digest 1: No. 5, October, 1937.

[2] Vaughan, W. T.: J. Allergy 6: 78, 1934; J. Lab. & Clin. Med. 21: 1278, 1936; J. Allergy 6: 421, 1935; J. Allergy 5: 601, 1934.

[3] Widal, F., Abrami, P., and Iancorusco, N.: Presse med. 28: 893, 1920.

Technic.—

The patient reports in the early morning without breakfast. After a quiet rest period of about 30 minutes, two fasting white counts are taken at 15- or 20-minute intervals, and their average is determined. (It goes without saying that all counts made on the same patient throughout the test must be made by the same person with the same white counting pipette, and counted in the same counting chamber.) The patient then eats an average portion of the food for which he is being tested. Total white counts are taken at 20-minute intervals for one and one-half hours. During the entire period of the test, the patient is not permitted to talk or move about.

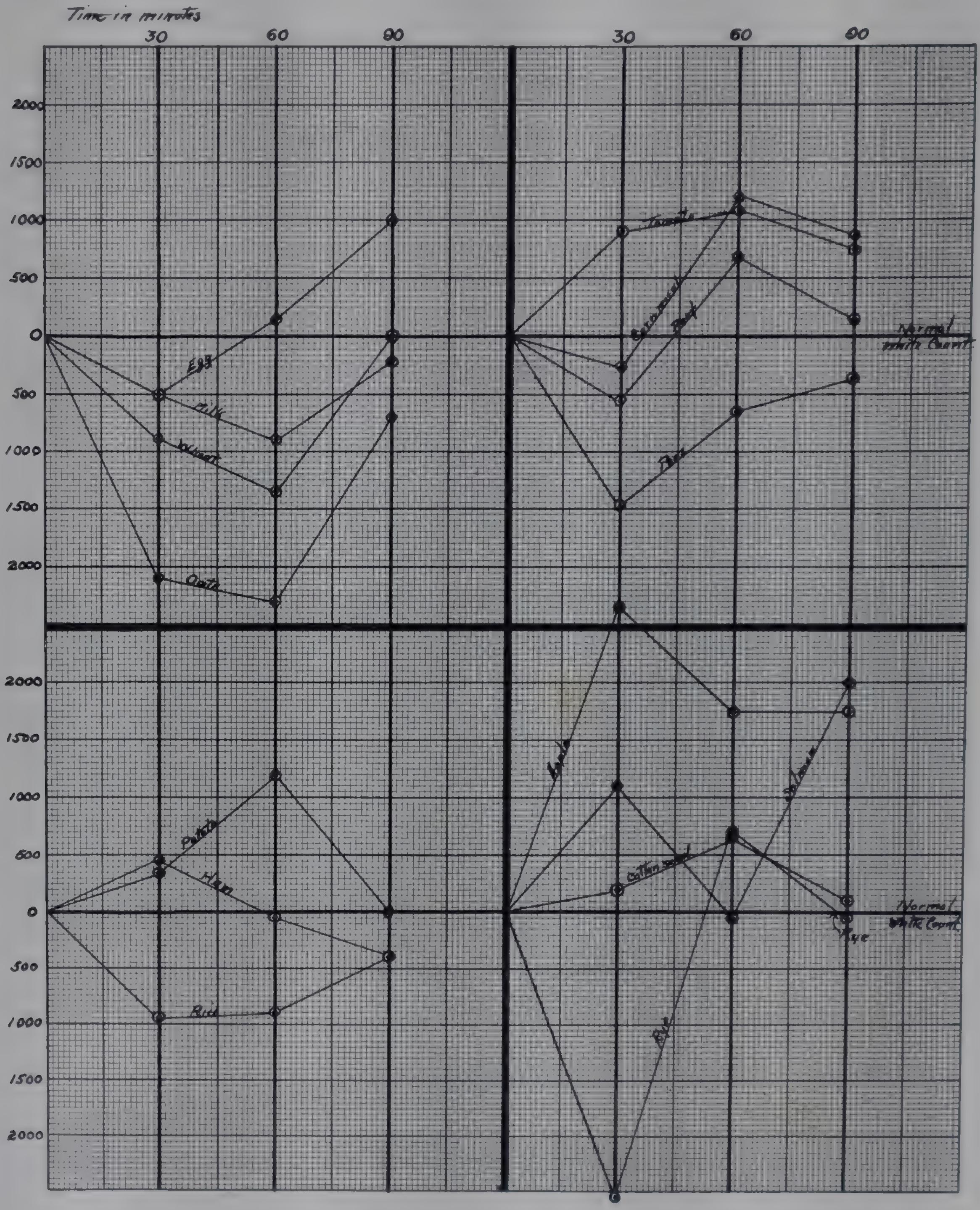

Fig. 322.—Leukocyte response to various foods. All curves dropping below the 1,000 mark are incompatible, or allergic responses. Those below the normal line, but showing a drop of 1,000 or less, are indeterminate, or borderline; those rising above the normal line are compatible or normal: Thus, egg, milk, cornmeal, beef, ham, and rice are indeterminate; while wheat, oats, peas, and rye are definitely allergic reactions. The other curves are normal. (Courtesy French K. Hansel, St. Louis.)

Using the average of the two first counts as the patient's normal leukocyte count, a graph is plotted to show the rise or drop in the white count throughout the test. Various types of curves have been noted. Hansel states that the type of curve is more important than the actual maximum drop or rise in the leukocyte count. They may be designated, as suggested by Rinkel and Gay,[4] as compatible, incompatible, or indeterminate. The compatible curve is one which shows a definite leukocytosis of 1,000 or more cells. This is a normal response. An incompatible curve shows a fall or leukopenia of 1,000 or more cells. This is a definite allergic reaction. The indeterminate curve may be of various types. First, there may be an initial leukocytosis followed by a definite leukopenia. Second, there may be an initial leukopenia followed by a leukocytosis. These indeterminate curves vary usually between a leukocytosis of 1,000 cells and a leukopenia of 1,000 cells. These are borderline cases.

According to Hansel, after the test has been completed, the patient should be questioned regarding the occurrence of symptoms, headache, itching of the skin, etc. The patient should record the foods eaten after the test so that upon the return visit it may be ascertained when the symptoms occurred after eating additional food.

In a statistical analysis of the leukopenic index, Sullivan reported his observations made in 500 tests performed at the Vaughan-Graham Clinic in Richmond, Virginia. In 89 per cent of this series of cases the variation between two fasting counts was no more than 500 cells, whereas 1.2 per cent of the cases had a variation of 2,000 or more cells. The amount of variation between the two fasting counts was definitely greater in those cases showing a high fasting count than in those with a low fasting count. It was pointed out that definite information may be obtained in 80 per cent of all cases in which the test is performed. An individual allergic to foods does not normally have a low fasting white count.

The determination of the leukopenic index has proved a very valuable procedure in the diagnosis of food allergy in its relation to respiratory, gastrointestinal, and other manifestations of allergy. The test is, coincidentally, also a constitutional test. The effect of the ingestion of the test food upon the production of symptoms also has great diagnostic value in many instances.

The test is particularly valuable in those instances in which the skin test is negative. In a study of 60 allergic patients on whom 191 leukocytic indices were made, Vaughan compared the results obtained with the skin reactions to the corresponding foods. In 118, or 62 per cent of the indices, the leukocyte responses corresponded with the skin reactions. In 87 the skin reactions were negative and there was no digestive leukopenia. In 31, the skin test was positive and ingestion of the food was followed by a digestive leukocytosis. The character of the skin test, therefore, confirmed the leukopenic index in 62 per cent of the cases. After including an additional 5.2 per cent which showed variable discrepancies, Vaughan concluded that the actual diagnostic effectiveness of the leukopenic index is 67.2 per cent.

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Zeller, M.: Illinois M. J. **69:** 54, 1936.

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[4] Rinkel, H. J., and Gay, L. P.: J. Missouri M. A. **38:** 182, 1936.

Dean, L. W.: J. A. M. A. 108: 251, 1937.
Jones, E., Stephens, D. J., Todd, H., and Lawrence, J. S.: Am. J. Physiol. 105: 547, 1933.
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Bronchial Secretions

The same set of facts pertaining to the observations on the cytology of the secretions of the nose and paranasal sinuses may be applied to the study of bronchial secretions; that is to say, the cellular content in the latter conditions should be given the same close study that we give nasal secretions and the same conclusions concerning allergic reactions may be drawn therefrom.

The eosinophilic and the eosinophilic-neutrophilic responses in bronchial secretions should be correlated with the clinical history and the physical findings. Often it is a question of whether or not an acute infection complicates an attack of bronchial asthma. Pure eosinophilic response would suggest a contact with some allergin or an eosinophilic-neutrophilic response would suggest either an acute or chronic infection, this to be determined by correlation of the cytology of clinical history, physical findings, etc.

Methods for the Study of Irritation and Toxicity of Substances Applied to the Skin and Mucous Membranes

Recent advances in chemistry and technology have led to the development of many new substances whose properties make them desirable for use in cosmetics. These include solvents, surface active agents, and new constituents for vehicles and bases. These solvents and wetting agents are capable of altering physical surface relationships between the absorbing medium (skin) and the substance to be absorbed. The division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C., in connection with its activities in World War II, examined the toxicity of several hundred compounds or preparations submitted as insect repellents, lousicides, or scabicides. This led to development of new methods. In the course of the tests additional data must be obtained from histopathologic examination, chemical studies on tissues and urine, and by physiologic function tests. Additional toxicity data on the preparation are obtained by administering it by other routes.[1, 2] Analysis of these data permits (a) an evaluation of the extent of injury, and (b) an estimation of the likelihood of repair.

These methods have been outlined by Draize.[3]

Test for Acute Toxicity.—

The rubber sleeve technic was developed specifically for testing the toxicity of hair waving solutions. It is also suitable for acute toxicity studies of any preparation intended for topical skin application. A sleeve made of rubber dam is so constructed that its ends fit snugly around the trunk of the animal. The body of the sleeve ''balloons'' slightly, furnishing a reservoir for the dose which is maintained without pressure in contact with the skin. The area of exposure represents 10 to 15 per cent of the total body surface. The sleeve is slipped onto the body of the animal which is then placed in a comfortable but immobilized

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[1] Woodard, G., and Calvery, H. O.: Indust. Med. 12: 55, 1943.

[2] Van Winkle, W., Jr., Herwick, R. P., Calvery, H. O., and Smith, A.: J. A. M. A. 126: 958, 1944.

[3] Draize, J. H.: Proc. Sci. Section of The Toilet Goods Ass'n, No. 5, May 17, 1946.

position in a multiple animal holder.[4] Preliminary doses on a body weight basis are introduced in the sleeve and allowed to remain in contact with the skin for varying periods of time (maximum of 24 hours). A sufficient number of animals is used to obtain mortality ratios at various doses. The dose which gives 50 per cent mortality (LD_{50}) may then be calculated.[5] Further analysis of the data enables the determination of the slope of the dosage-response curve and the probable error of a given determination.

At the end of the period of exposure the sleeves are removed, the volume of any unabsorbed material is measured, and the skin reactions are noted. The subjects are checked for gross symptoms of systemic effects and are transferred to individual metabolism cages where they are observed for a minimal period of two weeks. Body weight, food consumption, and gross symptoms of effects are checked daily. Observations for changes in blood morphology are made. Urine is checked for the presence of protein, reducing substances, blood pigments, or other abnormalities. The tissues of animals which die several days after exposure or those sacrificed because of severe poisoning are examined for histopathology.

Tests for Subacute Toxicity.—

In these tests, relatively large doses are applied by daily inunctions for periods up to twenty days to an uncovered area of the clipped intact skin. The size of the doses employed depend on the data obtained in the acute exposures, and may vary from 1.0 to 6.0 c.c./kg./day. The area inuncted is approximately 10 per cent of the total body surface of the animal. The material is gently rubbed into the skin with a glass rod and involves manipulation of the hair shafts and mild pressure. Animals are observed for two weeks following the last exposure.

The repetition of a dose, as in this type of experiment, may yield valuable information on the action of the agent on the skin and in the development of progressive systemic effects. In some cases there is gradual deterioration of the skin so that it becomes a less effective barrier as the doses are repeated. In other cases, the agent may produce a mummification or a coagulative effect in the skin so that absorption of subsequent doses of the agent becomes retarded.

The same observations and tests on the subjects are made as are performed in the experiments for acute toxicity. The urine and blood are examined, as in the above, and microscopic examinations of tissues of the severely poisoned subjects are made.

Further tests were made with an observation period of ninety days.

In these longer experiments for subacute toxicity, inunctions are made at dose levels of 0.5, 1.0, 2.0 and 4.0 c.c./kg. daily (Sundays excluded) for a 90-day period, to the clipped intact skin of animals. The areas treated with the material are approximately 10 per cent of the entire body surface. The conduct of these tests is similar to the 20-day experiments. The longer experimental period enables observation of the slow progressive deterioration produced by some agents after prolonged administration. Special emphasis is placed on blood morphology to ascertain the possible effect of the agent on the hemopoietic organs or on the blood itself. The tissues of all animals on these experiments are examined for histopathology.

The toxicity of more than 500 compounds or preparations intended for topical skin application have been studied in Draize's laboratory during the past five years.

The acute toxicity data (LD_{50}) for a few selected compounds administered orally and by topical skin application to the rabbit are given in Table 137.

The data in Table 137 illustrate that compounds differing widely in chemical structure and behavior may be readily absorbed by the skin. There does not appear to be a consistent relationship between toxicity following the oral and the topical route of administration. Although the LD_{50} is lower for the oral route in the case of a large majority of the compounds, occasionally a compound may exhibit a lower LD_{50} when administered by the topical skin route.

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[4] Laug, E. P.: J. Lab. & Clin. Med. 29: 308, 1944.

[5] Bliss, C. I.: Quart. J. Pharm. & Pharmacol. 11: 192, 1938.

TABLE 137.—TOXICITY FOR RABBITS OF SOME ORGANIC COMPOUNDS

	LD_{50}	
COMPOUND	ORAL (ACUTE)	SKIN (ACUTE)
1. Beta-butoxyl-beta thiocyano diethyl ether	0.03	0.25-0.50
2. Beta thiocyano ethyl esters of fatty acids C_8—C_{18}	0.05	5.0 (C_{16} acid, ester)
3. Isobornyl thiocyanoacetate	0.63	10.0
4. 2-Ethyl-1,3 hexanediol	2.6	10.0+
5. Diethylene glycol monobutyl ether-acetate	2.6	5.5
6. Diethylene glycol mono ethyl ether	2.75	7.5
7. 2-Methyl-2,4-pentanediol	3.25	9.0
8. Diethylene glycol	4.4	10.0+
9. Dimethyl phthalate	4.4	10.0+
10. Dimethyl gamma pyrone-butyl ester	5.3	10.0+
11. 2-Ethyl butanol	1.2	1.5
12. Dibutyl oxalate	2.5	0.5

Some compounds which appear to be relatively nontoxic on acute exposure to the skin may upon repeated application (inunction) be severely damaging. Compounds which behave in this manner often produce no gross skin damage with the first exposure but produce severe skin damage (even to necrosis and desquamation) upon repeated exposure. The regenerated skin following damage is often more resistant to the future action of a given compound.

Primary Irritation.—

A modified patch test technic on the intact and abraded skin of the albino rabbit is used to measure primary irritation of the skin. Four areas on the back, approximately 10 cm. apart, are designated for the position of the patches. The skin is clipped free of hair, and in the area of two of the four patch positions, four epidermal incisions (two perpendicular to the other two) are made to produce an abraded skin. The patches consisting of two layers of gauze cut in squares (2.5 cm. on the side) are secured in position by thin bands of adhesive tape. The material to be tested is introduced under the patch—0.5 c.c. in the case of liquids and 0.5 gm. for solids. A balanced pattern is used for distribution of the four preparations to be tested on a series of six rabbits. This pattern is such that each compound appears once on a given subject. Three applications to intact skin and three to abraded skin are made for each preparation. The entire trunk of the animal is wrapped in rubberized cloth, which helps to hold the patches in position and retards evaporation of volatile substances during the 24 hours of exposure. The animals are immobilized in the special holder during the 24-hour patch exposure. Upon removal of the patches the skin reactions are assigned scores in proportion to their severity. Readings are also made after 72 hours and the final score represents an average of the 24- and 72-hour readings. The table of scores evaluates the erythema and edema produced, giving each factor equal weight. Maximal reactions are given values of 4 respectively. No values are given for agents which elicit severe escharotic or vesicant action. When such an agent is encountered a solution of it in a bland nonirritating solvent is prepared. Sufficient dilution is prepared so that the response may be graded by the scale.

Compounds producing combined averages of 2 or less are mildly irritating, whereas those with indexes of 2 to 5 are moderate irritants and those with scores of 6 or more are considered severe irritants.

Using this technic Draize's laboratory has determined the index of primary irritation to rabbit skin for approximately four thousand compounds or preparations during the past five years.

In general, compounds with similar chemical structures have a tendency to exhibit similar degrees of irritancy. There are, however, many exceptions. These exceptions are sufficiently numerous to warrant the conclusion that potential skin irritation may not be predicted solely on the basis of chemical structure.

TABLE 138.—INDEX OF PRIMARY IRRITATION (RABBIT)

COMPOUND	SCORE
Dibenzyl ketone	1.0
Isobutyl benzyl ketone	1.3
Methyl isobutyl ketone	1.7
Di-isobutyl ketone	8.0
Alpha tetralol (1,2,3,4-tetrahydro-1-naphthol)	2.3
Beta tetralol (1,2,3,4-tetrahydro-2-naphthol)	3.7
Alpha decalol	7.7
Beta decalol	3.7
1-Methyl (1,2,3,4-tetrahydro-2-naphthol)	1.3
1-Ethyl (1,2,3,4-tetrahydro-2-naphthol)	2.3

In Table 138 are given data on the primary irritation of a series of closely related ketones and naphthyl derivatives. It will be noted that compounds which have closely related chemical structures may exhibit very different indexes of skin irritation.

Primary Irritation of Mucous Membrane.—

This is measured on the rabbit eye and penis. In the measure of injury to the eye a modification of the scoring system used by Friedenwald et al.[6] was devised. Injuries to the cornea, iris, and the conjunctival and palpebral mucosae are scored separately.

Technic.—

0.1 c.c. of the substance is instilled in the conjunctival sac. Readings are usually made at 1, 24, and 48 hours after instillation. Injuries to the cornea are graded on the degree of opacity produced and total area involved. Injuries to the iris are graded on the extent of congestion, swelling, and its ability to react to light. The conjunctivae are graded on the degree of redness, chemosis, and the amount of discharge. In scoring the eye for over-all effect the maximal injury for cornea, iris, and conjunctiva receive scores of 80, 10, and 20, respectively.

These technics enable one to describe the extent of toxicities. In many instances a preparation may be recognized as unsafe following a few tests. In other cases injuries are insidious in that they develop much more slowly and require more complete investigation than this before a decision can be reached concerning their safety for use.

SEMEN APPRAISAL

The examination of seminal fluid with special regard to defective spermatogenesis in otherwise healthy men has been elaborately studied by Cary and Hotchkiss.[7] They present a method of fixing and staining a microscopic specimen which differentiates component parts of the cell without distorting the protoplasm and enables any physician familiar with the use of the oil immersion lens to recognize and classify abnormal sperm cells, and, in specimens of doubtful character, to count the percentage of these cells as an added index of deficiency or improvement.

The examination of the semen is divided into three steps: (1) collection of the specimen; (2) the gross or macroscopic investigation; (3) the microscopic study.

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[6] Friedenwald, J. S., Hughes, W. F., Jr., and Herrmann, H.: Arch. Ophth. 31: 279, 1944.

[7] Cary, W. H., and Hotchkiss, R. S.: J. A. M. A. 102: 587, 1934.

1. **The Collection of the Specimen** may be accomplished by use of any one of the three following methods, with preference in the order named.

(a) A specimen produced at the office of the examiner. If accuracy is stressed, and a quiet, suitable room is provided, many men are not averse to this method, and the physician is in constant control of the ejaculate.

(b) Collection outside the office by external emission. For this purpose we lend the patient a glass graduate and a small (1 ounce) wide-mouth bottle. After collection in the glass graduate, specimen is allowed to liquefy so that it will not adhere to the glass surface, and it is transferred to the bottle, which is then corked, placed under the clothing about the torso, and brought to the office.

(c) Condom Method. Patients are advised to prepare the condom in advance by washing the preservative powder from the surface that is to receive the specimen, carefully drying it. This precaution is taken because not only do foreign bodies hinder the microscopic examination, but there is a chance of harm to the specimen by the various ingredients in these powders. After coitus the condom is tied, gently wrapped in a handkerchief (not clinging, absorbent cotton), concealed under the garment next to the skin, and thus delivered.

Patients are warned not to attempt artificial warming of the specimen; but gentleness in handling and maintaining, as far as possible, an even body temperature should be borne in mind. The laboratory should be ready for examination of the specimen when it arrives. The necessary implements, cleansed in plain hot water and thoroughly dried, should be kept ready for the examination. At the appointed hour, all the apparatus should be ready, including the microscope, to avoid thermal shock to the semen and the annoyance of misty lenses.

2. **Gross Examination** includes a notation of the amount, which, when complete, and from men under forty years of age, should exceed 3.5 c.c. Lesser amounts should arouse suspicion of deficiency and premature weakening by vaginal acidity, otherwise negligible. Extended periods of sexual abstinence may impart a slightly yellow tinge to the normal grayish opaqueness. Normal specimens commonly show a slight degree of viscosity, which is easily demonstrated by slowly expelling a drop of semen from a pipette. Absence of viscosity with a lessening of opaqueness points to reduced cell content, although the reverse deduction does not hold.

An alkalinity of pH 8.1 to 8.4 has been so constant that it seems an unnecessary refinement except in research study; the significance of wider variations in reaction is not yet known. It has been pointed out also that the loss of carbon dioxide alters the body chemistry of the specimen.

3. **Microscopic Examination.**—Two specimens, one thick and one thin, are promptly prepared for microscopic examination by gently protecting a full drop and a fractional drop with cover glasses, the former permitting a gross impression of the density and motility of the cellular content, the latter allowing a preliminary study of individual cell morphology. In the thicker preparation, semen of relatively high fertility presents a field teeming with spermatozoa, many of which are rapidly propelled by fast-whipping tails. While universal cell activity would constitute the ideal, one usually notes in comparatively vigorous specimens that these fast travelers are colliding with a certain number of inactive (average 25 per cent), and a variable number of sluggish and impotent cells, which gradually sink toward the lower strata of the field. Familiarity with this picture under standard, dry, high-power magnification constitutes the measure by which motility is judged. It is a practical observation that a motile field, though definitely subnormal, is apt to convey a favorable impression on the inexperienced or occasional examiner, but reverse errors in interpretation are rarely made. The practical aspects of motility are that a large number of highly motile cells are essential to fertility, and when a specimen of normal quantity and rich cellular content shows 25 per cent or more of these dynamic cells, a rating of relative fertility must be assumed unless strongly disproved by other indices to be described. Such specimens usually meet other major requirements of fecundity.

Richness of cellular content, in terms of millions per cubic centimeter, has been given emphasis by Macomber, Meeker, and other workers as a further index of appraisal. While significant degrees of oligospermia are usually apparent to the experienced examiner on microscopic examination, there is no verbal picture that will adequately provide such an index. It may be said, however, that such specimens usually present other evidences of subnormality, such as lowered motility, increased number of abnormal cells, early crystal formation, and sometimes considerable indescribable debris. Such specimens may contain several round, granular cells in each field. These may be leukocytes or cells from the seminiferous tubules. The larger granular cells are spermatocytes; there are usually four nuclei, which may be demonstrated by the staining process later described. Cell counts are probably modified by the amount of the seminal fluid, and, while in agreement with other observers that counts above 70,000,000 per cubic centimeter are found in fertile specimens, Cary and Hotchkiss hesitate in setting a medium arbitrary count below which fertility is impossible. On the other hand, they have found a count as high as 112,000,000 in a highly defective specimen which measured less than 20 minims total quantity. This specimen promptly improved after treatment, and pregnancy followed.

Duration of motility is one essential to fecundation that can be easily determined. For this purpose the edge of the cover glass on the thicker smear should be protected by a layer of petrolatum or new smears should be made from hour to hour from the total specimen. A normal specimen exhibits little cessation at the third hour after emission, and considerable cellular activity should be found at the fifth or sixth hour. Complete or considerable subsidence of motility within this limitation should be regarded as a further index of deficiency, but it should be noted that the few motile cells that were originally active in a highly defective specimen occasionally survive for many hours. Reciprocal studies between the direct male specimen and its behavior in the female secretions tend to indicate that duration is practically synonymous with early vigor of motility; but there may be exceptions to this rule.

These writers believe that there is an urgent need for an improved staining process to define more clearly the exact morphology of the spermatozoon.

After many trials with various agents it was found that Schaudinn's solution was ideal for the fixation process, for it not only preserved the normal morphology of the cells, but also removed the objectionable débris. The next problem was to devise a balanced stain that would demonstrate all portions of the spermatozoon, yet spare the chromophilic substance from overstain.

The following technic permits illustration of the head, midsection, and tail of the sperm:

A. Prepare thin cover slip smears.

B. Fixation in Schaudinn's solution:

1. Immerse for one minute in 7 per cent solution of corrosive mercuric chloride, 2 parts and absolute alcohol, 1 part.
2. Immerse for half a minute in 50 per cent alcohol.
3. Immerse for half a minute in distilled water, 3 ounces, and tincture of iodine, 2 drops.
4. Wash in tap water.

C. Staining process:

1. Immerse for half a minute in aqueous solution of eosin, 5 per cent.
2. Immerse for one minute in 50 per cent alcohol, 3 ounces, and concentrated hydrochloric acid, 2 drops.
3. Wash in distilled water.
4. Immerse for two and one-half minutes in hematoxylin.
5. Immerse for one minute in distilled water, 3 ounces, and glacial acetic acid, 2 drops.
6. Wash in distilled water.

Dry and mount.

In appraising seminal specimens, deduct the percentage of abnormal forms found from the total cell count. The resultant figure, when correlated with other data, is of great importance in the more accurate evaluation of the semen. For instance, 40 per cent of inactive cells with 25 per cent abnormal forms would indicate more serious impairment and need for greater improvement in a specimen with a count of 60,000,000 or less than would obtain with a cell count of 100,000,000.

The Weisman Method of Counting Spermatozoa

Weisman* uses a simple method which obviates the necessity of blood counting pipettes and special diluting fluid. Dilution with ordinary tap water serves not only to dilute the sperm cells so that they may be easily counted in a Neubauer blood-counting chamber, but it also causes immediate cessation of motion of the sperms, facilitating the count.

Technic.—

Place 1 c.c. of well-mixed whole semen in an ordinary test tube, graduated at 20 c.c. Add ordinary tap water, cold, up to the 20 c.c. mark.

This technic kills all the spermatozoa at once and by dilution of 1:20 diminishes the error which occurs when using the small amounts of semen usually utilized in the blood-counting pipette technic.

Fill the counting chamber. Count the sperms in the same fashion as white cells (pages 575 ff.). Count and recheck the sperms in 1 sq. mm. (the sixteen small squares in one corner of the chamber); multiply by 10 to obtain the number of sperms found in 1 cu. mm. Multiply this by 1,000 to find the number in 1 c.c. of diluted semen. To obtain the number in 1 c.c. of whole undiluted semen, multiply this by 20. In other words, multiply the actual average count in the 1 sq. mm. field by 2 and add five zeroes.

MEDICOLEGAL EXAMINATION OF SEMINAL STAINS

The identification of seminal stains is frequently of great value in medicolegal work in cases of alleged rape, sexual homicide, or even adultery. Specimens are sent to the laboratory often dried on clothing, sometimes admixed with blood, sometimes in dry masses adherent to hair. Spots on the specimen may be found very readily if submitted to examination by ultraviolet light. Seminal stains give a greenish white fluorescence which may aid in finding the stained areas. This fluorescence is not specific for semen.

Seminal stains are detected definitely by finding spermatozoa under the microscope. The spermatozoa are not readily destroyed unless the stain has been subjected to too much washing or exposure. After they become dry they are very brittle, and the tail is easily separated from the head.

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*Weisman, A. I.: J. Lab. & Clin. Med. 27: 669, 1942.

To identify them absolutely, it is necessary to find perfect bodies with head and tail complete. It is best if motility can be shown (only possible when specimen is fresh and moist). Great care must be exercised in handling stains that are to be examined for the presence of spermatozoa.

If it is possible to obtain a fresh specimen, spermatozoa may be found by putting a small sample of the semen in a drop of physiologic saline and examining microscopically; motility may be found in recent specimens. The slide may be stained by various methods.

The spermatozoa, or male germ cells, are developed in the convoluted tubules of the testes. They are actively motile in the seminal fluid, which is a secretion of the accessory sexual glands, and are seen in enormous numbers in the normal ejaculate. A spermatozoon is a slender elongated flagellated structure 50 to 70 microns in length and possesses a head, neck, and body or connective piece, and a tail.

The head is pear-shaped, measuring from 3 to 6 microns in width. The neck consists of the anterior centrosome; the body is rodlike and connects the head and neck with the tail. The tail is divided in two portions, the chief piece and the end piece.

Spermatozoa are actively motile in the fresh state.

Seminal fluid or semen is a mixture of secretions of the epididymis, the ductus deferens, the seminal vesicles, the prostate, the bulbourethral or Cowper's glands, and the urethral or Littre's glands. Average amount of ejaculate is 2.5 to 5 c.c., and each cubic centimeter should contain 100,000,000 or more spermatozoa.

Methods of Staining Spermatozoa

Method of Cary and Hotchkiss[1] (1934).—

A. Prepare thin cover-slip smears as used in the preparation of blood for staining.

B. *Fixation in Schaudinn's solution:*

While still wet immerse for one minute in a 7 per cent corrosive mercuric chloride, two parts, and absolute alcohol, one part. Immerse for half a minute in 50 per cent alcohol. Immerse for half a minute in distilled water, 3 ounces, and tincture of iodine, 2 drops. Wash in tap water.

C. *Staining process:*

Immerse for half a minute in a 5% aqueous solution of eosin. Immerse for one minute in 50 per cent alcohol, 3 ounces, and concentrated hydrochloric acid, 2 drops. Wash in distilled water. Immerse for two and one-half minutes in hematoxylin. Immerse for one minute in distilled water, 3 ounces, and glacial acetic acid, 2 drops. Wash in distilled water. Dry and mount.

This method gives excellent contrast between the nuclear material, which stains blue, and the cytoplasm, which stains red.

Method of Meaker.[2]—

Fix with heat. Wash with 0.5 per cent Chlorazene, to remove mucus. Wash with water. Wash with 95 per cent alcohol, and dry. Stain with carbol-fuchsin and bluish eosin (carbol-fuchsin two parts, filtered bluish eosin one part, alcohol one part). Wash with water. Stain with methylene blue (methylene blue one part, water four parts). Wash with water.

Method of Gelarie.[3]—

This is a one-minute method for the staining of spirochetes, spirilla, spermatozoa, and related organisms.

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[1] Cary, W. H., and Hotchkiss, R. S.: Semen Appraisal, J. A. M. A. 102: 587, 1934. Weisman, Abner I.: Spermatozoa and Sterility, p. 59, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

[2] Meaker, S. R.: Human Sterility, Baltimore, Williams and Wilkins, 1934. Weisman, Abner I.: Spermatozoa and Sterility, p. 59, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

[3] Gelarie, A. J.: A New, One Minute Method for Staining Spermatozoa, etc., Am. J. Obst. & Gynec. 21: 1065, 1936. Weisman, Abner I.: Spermatozoa and Sterility, p. 60, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

Preparation of the solutions:

Fixing Solution.—Dissolve 2.5 gm. of recrystallized zirconyl chloride ($ZrOCl_2$) in 100 c.c. of 10 per cent sodium chloride solution.

Citric Acid Solution.—Make a 10 per cent solution of recrystallized citric acid in distilled water to which a few crystals of thymol are added to avoid fungous formation.

Staining Solution.—Dissolve 0.25 gm. of gentian violet in 100 c.c. of N/10 solution sodium lactate. The pH of this mixture should be approximately 8.4; if it is not, it should be so adjusted.

Solution of K_2HgI_4.—Dissolve 0.5 gm. of mercuric iodide in 100 c.c. of 0.5 per cent potassium iodide solution.

Counterstain.—Dissolve 0.2 gm. of methylene blue in 100 c.c. of distilled water and add 0.2 gm. of carbolic acid. (For photographic purposes use aqueous carbol-fuchsin instead of methylene blue.)

Technic.—According to Gelarie, the material to be examined is smeared on the slide in a thin layer and allowed to dry in the air (not over the flame). Cover with the zirconyl chloride solution, allow to stand for five seconds, and then wash with water. This acts as a fixative. Then place a few drops of the 10 per cent citric acid solution on the smear, allow to remain for ten seconds and wash again with water. The slide is now ready for staining. Place a few drops of the staining solution on the slide, allow to remain for thirty seconds, and then wash off with water. Follow by momentarily covering the smear with a few drops of the complex mercuric iodide solution and again wash with water. While a good stain is obtained at this point, uniformly excellent results may be secured by counterstaining with aqueous carbol methylene blue solution for five seconds. Then wash the slide, dry, and examine by means of the oil immersion lens. Counterstaining is not recommended for obtaining exact detail of spermatozoa, however, due to overstaining of the organism. Without it the different parts—head, neck, and tail—with their components can be readily distinguished.

Although the above technic is simple, it is believed that some of the more complicated stains show up much more detail of the spermatozoal head.

Method of Holbert.[4]—Holbert's method is similar to that used by Williams: Make a thin smear like a blood smear. Shake until dry. Flame gently. Flood slide with 0.5 per cent Chlorazene for two minutes. Wash gently with running water. Flood slide with 95 per cent alcohol. Let stand for one minute. Tip off excess alcohol and allow to dry. Stain with 0.5 per cent aqueous gentian violet for three minutes. Rinse with water. Rinse with 95 per cent alcohol. Rinse with water. Counterstain with 1 per cent aqueous rose bengal for one minute. Wash with water. Allow to dry. **This method is recommended.**

Methods of Williams and his co-workers.[5, 6]—

Williams has three methods of obtaining spermatozoa. The smear is made in the same way as a blood smear, dried in the air, fixed with moderate heat, and treated with 0.5 per cent Chlorazene solution for two to four minutes to remove the mucous film that overlies the spermatozoa. Wash slide gently with distilled water and then with 95 per cent alcohol. Stain as follows:

Method I. Crystal violet and rose bengal.—

Cover film with 0.25 per cent aqueous solution of crystal gentian violet for two to three minutes. Wash with water. Decolorize with 95 per cent alcohol for half a minute to two minutes. Wash with distilled water. Counterstain with 1 per cent rose bengal (aqueous) for eight seconds. Wash in water and examine under microscope.

If the spermatozoa have not taken up sufficient rose bengal to impart a distinct reddish color to the acrosome and the nuclear membrane, repeat the staining interval with rose bengal until this is accomplished. Care should be taken not to overstain with rose bengal; if overstained, the contrast between the nucleus and the acrosome will be lost.

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[4] Holbert, P. E.: J. Lab. & Clin. Med. 22: 320, 1936. Weisman, Abner I.: Spermatozoa and Sterility, p. 60, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

[5] Williams, W. W., McGugan, A., and Carpenter, H. D.: J. Urol. 32: 201, 1934. Weisman, Abner I.: Spermatozoa and Sterility, p. 61, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

[6] Williams, W. W.: New England J. Med. 217: 946, 1937. Weisman, Abner I.: Spermatozoa and Sterility, p. 61, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

Method II. Wollschwarz, methylene blue and rose bengal:

Stain in 1 per cent solution of Wollschwarz for five minutes as follows:

Prepare three solutions: A, B, and C.

	A	B	C
Wollschwarz (1 per cent aqueous)	4.0 c.c.	4.0 c.c.	4.0 c.c.
Sulphuric acid, 2 per cent	0.025 c.c.	0.05 c.c.	0.075 c.c.

The smear on the microscope slide is marked off into three divisions with a glass-marking pencil, and the solutions of stain having the different concentrations of sulphuric acid are applied to each of the divisions, respectively. (Dilutions A and C may be omitted.) Wash with water. Counterstain for six to eight seconds with Loeffler's methylene blue that has been diluted in fifteen parts of water. This acts as a mordant to the Wollschwarz, serving to fix it in the nucleus. It imparts a rather greenish hue to the otherwise yellowish-stained nucleus. Wash with distilled water. Counterstain with 1 per cent aqueous rose bengal for six seconds, or until the rose bengal is set in the acrosome and the nuclear and cell membranes to give the desired contrasts. Examine slide after each application of stain so as to guide its intensity. Wash with distilled water. Dry, mount in neutral Canada balsam, and apply coverslip, or examine without mounting.

Method III. Carbol-fuchsin, eosin-B, and methylene blue:

Prepare the two following staining solutions:

Stain 1.	Saturated alcoholic solution of eosin (bluish)	1.0 c.c.
	Ziehl-Neelsen carbol-fuchsin	2.0 c.c.
	Alcohol 95 per cent	1.0 c.c.

Mix the eosin and carbol-fuchsin solutions together in a dry watch glass. Immediately on mixing, an insoluble precipitate and a metallic film form. Filter the mixture, and to the filtrate add 1 c.c. of 95 per cent alcohol.

Stain 2.	Loeffler's methylene blue	1 part
	Distilled water	6 to 8 parts

Apply Stain 1 to the smear for two minutes. Wash film gently with distilled water. This is best done by immersing it in a basin. Counterstain with Stain 2 for four to eight seconds. Wash slide with distilled water, and dry.

Method of Pollák and Joël.[7]—

Use two separate smears and stain them differently. One stain brings out the structure of the entire spermatozoon, and the second affords better visualization of the cell structure. Thus the stains complement each other.

Stain I. Hemalum-eosin (for visualizing spermatozoal structure). Fix for two minutes with methyl alcohol and then rinse in water. Stain for twenty minutes with hemalum,* steep in running water for thirty minutes with fresh hemalum, rinse for ten minutes in water, stain for contrast for three minutes with a 3 per cent alcoholic eosin solution, differentiate in an alcohol sequence of 70, 90, and 100 per cent, clear in carbolxylene, and embed in Canada balsam under a cover glass.

Stain II. Azure-eosin (for the cellular structure). Fix for two minutes with methyl alcohol, stain for twenty minutes with a diluted Giemsa-Romanowsky azure-eosin solution, rinse in distilled water, and embed in Canada balsam under a cover glass. The staining solution is prepared fresh each time; to 5 c.c. of distilled water add ten drops of the Giemsa-Romanowsky solution.

Examination for Spermatozoa in Seminal Stains

If the stain is such that a fragment can be removed with the point of a knife, the examination is fairly easy. Remove a portion of the scaly stain with a knife point and place it on a glass slide on which has previously been placed a drop of distilled water. Allow the stain to soak in the water for several hours. Keep the specimen moist under a bell jar or in a large closed Petri dish. Separate the scales very cautiously with a teasing needle, allow to dry, fix in methyl alcohol, and stain with dilute Giemsa stain (1 drop in each c.c. of neutral distilled water) for thirty minutes, repeating the stain for an additional thirty minutes if necessary. If restaining is attempted, dilute fresh stain. Wash gently with distilled water, stand the slide on end, and allow to dry. Examine for spermatozoa under the oil immersion objective.

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*Hemalum is similar to hematoxylin, but not identical. It is a 2 per cent solution of crystalline hematin in 95 per cent aqueous solution of $KAI\ (SO_4)_2.12\ H_2O$, 100 c.c.

[7] Pollák, O. J., and Joël, C. A.: J. A. M. A. 113: 395, 1939. Weisman, Abner I.: Spermatozoa and Sterility, p. 62, Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros.

If the stain is on unstarched cotton or linen cloth, the finding of spermatozoa presents a much more difficult problem. Select a thread of the cloth near the center of the stain, select the side of the cloth which contains the more seminal fluid. Place a thread from the dry stain on a glass slide in a drop of distilled water. Make many such preparations. Each specimen is allowed to soak in the water for from two to twenty-four hours. Provision must be made to prevent drying of the water during this time.

At the end of the digestion period tease the fibrils apart *very cautiously* with fine pointed needles. Take care not to break the spermatozoa. Continue with the staining process just described.

The cloth itself may be stained according to the **method of Baecchi.**[*] A piece of the cloth containing the stain is cut 1 cm. square, and placed in 1 per cent acid fuchsin or methylene blue solution. It is then washed in 1 per cent hydrochloric acid, after which it is dried in the air or dehydrated in absolute alcohol. Next it is cleared in xylol, then mounted in Canada balsam. If the spots are not fresh, they are placed for one-half hour to twenty-four hours in 20 to 30 per cent ammonium hydroxide solution and then in distilled water preliminary to the staining process.

Hektoen and Rukstinat[**] describe a **method** of making a stained specimen from spots of spermatozoa on cloth. Their method makes use of Mallory and Wright's chloride of iron-hematoxylin stain.

1. Soak the stained cloth in 1 per cent ammonium hydroxide solution in water for from twelve to twenty-four hours.
2. Select a single thread and stain two minutes in 5 per cent aqueous solution of erythrosin.
3. Wash quickly in distilled water.
4. Stain with 10% aqueous solution of ferric chloride two minutes.
5. Transfer directly to 2% aqueous solution of hematoxylin for two minutes.
6. Wash quickly in distilled water.
7. Transfer to 0.5% aqueous ferric chloride and tease out fiber to its finest shreds. Remove from this solution when threads are reddish pink or light brown.
8. Wash in distilled water quickly.
9. Dehydrate in absolute alcohol.
10. Clear in creosote.
11. Mount in balsam.

Better results are obtained if the differentiation is carried out slowly in 0.5% ferric chloride solution than in stronger solutions. Boiling the cloth for one minute in water after soaking in ammonia solution helps preserve the tails. When the stain takes well, the head of the spermatozoon is from a dark purple to black in the posterior two-thirds, while the anterior third is pink, and the tail is from a pink to lavender. The connecting piece frequently is evident. The threads of the cloth may stain more or less pinkish.

Weisman[1] refers directly to the technic of Hankin[2] and Windsor.[3] This is a special technic for warm climates. Hankin examined the suspected stains directly on the cloth, after first fixing the material with a cellular fixative, then adding dilute sulphuric acid, and finally staining the material with gentian violet. Windsor fixed the cloth in Müller's solution and then stained it. Williams suggested the special Wollschwarz[1] stain. Soak and scrape the semen-stained cloth, obtaining a turbid suspension. Centrifuge the soakings. In an old specimen, some of the tails will be missing or bent or abnormally twisted. Care must be taken not to confuse the sperm heads with spores, pus cells, or artefacts.

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[*] Vierteljahrsschrift f. gerichtl. Med. 34: 1, 1912.

[**] Hektoen, L., and Rukstinat, G. J.: Arch. Path. 1: No. 1, 1928.

[1] Weisman, Abner I.: Spermatozoa and Sterility, p. 210, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

[2] Hankin, E. H.: Brit. M. J. 2: 1261, 1843, 1906.

[3] Windsor, F. N.: Brit. M. J. 2: 501, 1908. Weisman, Abner I.: Spermatozoa and Sterility, p. 210, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

The Florence Test for Identification of Semen.—

Reagent.—

Potassium iodide	1.65 gm.
Iodine	2.54 gm.
Distilled water	30.00 c.c.

Rub up the iodine and the potassium iodide in a mortar, then add the water. Filter before using.

Technic.—

Remove a minute fragment of the stained specimen by means of a pair of fine-pointed forceps and sharp-pointed scissors. Transfer to a slide. Treat with a small drop of distilled water. Allow to soak for 1 or 2 minutes. Add a small drop of the reagent to it in such a way that the two drops of fluid come in contact at their edges, and then cover immediately with a cover glass.

Examine microscopically for seminal crystals. These look like the hemin crystals obtained with Teichmann's test for blood (Fig. 292, page 1204).

Weisman[2] states that a positive reaction is not significant since other body fluids give such results with this test. Our own experience confirms this statement. A negative test, however, informs the investigator that the suspected substance is not semen, and this is the only value of the Florence test.

Peltzer[3] uses the following technic for the Florence method:

Moisten the suspected spots with hydrogen peroxide. If semen is present, much foaming occurs. Centrifuge substance. Place on slide and stain with 2 per cent aqueous eosin. If positive, long lance-like crystals appear. The addition of I-KI solution to the slide colors the crystals brown. The crystals disappear and are recrystallized by the addition of more I-KI solution. The presence of Florence crystals denotes the presence of body fluids, possibly semen.

The Puranen Test.—Puranen[4, 5] and Tarsitano[6] advocated the microchemical method for identification of spermatozoa. Naphthol yellow sulphur in aqueous solution reacts differently with human semen at neutral and slightly alkaline reactions. A positive reaction is denoted by formation of microcrystals, which are characteristically large, have a strong double refraction, and are of a definite orange hue.

Niederland's Test.[7]—This test employs dilute sulphuric acid. When added to a suspected specimen, the weak sulphuric acid solution is always positive in the presence of semen, but this test is also positive with practically all other body secretions. Weisman states that its only value lies in the fact that, when the test is negative, further search for spermatozoa can be abandoned. The crystals formed are probably sulphates of calcium and other salts found in semen and body fluids.

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[1] Loc. cit., page 1368.

[2] L. c., footnote p. 1370.

[3] Peltzer, J.: Chem. Ztg. 55: 70, 1931. Weisman, Abner I.: Spermatozoa and Sterility, p. 211, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

[4] Puranen, U. H.: Acta chem. Fennica. 8B: 7, 1935.

[5] Puranen, U. H.: Deutsche Ztschr. f. d. ges. gerichtl. Med. 26: 366, 1936.

[6] Tarsitano, F.: Diag. e tec. di lab. 8: 353, 1937.

[7] Niederland, W.: Med. Welt. 5: 149, 1931.

Marcille Method.[7]—This is nothing more than the Florence test coupled with the addition of Gram-eosin staining of the centrifuged precipitate after maceration of the specimen in physiologic saline solution.

Borgarius Test.—This test is similar to the Florence test, except that phosphomolybdic acid solution instead of the I-KI solution is used to precipitate crystals. The crystals obtained by this technic are characteristic, but they are also found with other body fluids.

The AuI_3 Test.—Villamil[8] has shown that solutions of AuI_3 give characteristic crystals with diluted semen as well as with solutions of spots and stains produced by semen. The crystals are distinct. He considers this reaction to be more sensitive than that obtained by most other microcrystalline tests.

Test for Hydrogen Ion Concentration.[9]—If a drop of distilled water is added to suspected semen and well mixed, the hydrogen ion concentration of the solution when tested with nitrazine paper and compared with a colorimetric chart will give evidence of definite alkalinity.

The Fluorescence or Ultraviolet Light Test.—Ito[10] first demonstrated that semen stains give a characteristic color effect under ultraviolet light. He obtained this same effect with other tissues and described its use in forensic medicine.

Simonin[11] showed that dried sperm gives a brilliant characteristic fluorescence under ultraviolet light, but stated that other organic liquids also give some fluorescence, so that the test is probably not specific. Husson[12] reported that seminal spots, irrespective of spermatozoa or azoospermia or the age of the spot, show a characteristic fluorescence in ultraviolet rays between 4200 and 4900 Å.

Microchemical Identification of Seminal Fluid

Method of Choice

Determination of acid phosphatase activity affords an excellent means for specific microchemical identification of seminal fluid and seminal stains. This procedure employs a new principle postulated by Ove Riisfelt of Denmark in 1946.* It is of definite medicolegal value in the identification of seminal fluid as an adjunct to the demonstration of spermatozoa; but it is of particular value in cases where spermatozoa cannot be demonstrated in the specimen.

Kaye** found that male ejaculate produces a very high acid phosphatase activity in comparison with the very low acid phosphatase activity of the other body fluids (saliva, perspiration, urine, etc.) and the common vegetable and fruit juice stains. The differences are so marked and definite that it leaves little room for doubtful interpretation of the results. The average seminal fluid contains approximately 2,500 King-Armstrong units of acid phosphatase per c.c., the upper limits ranging to 3,500 King-Armstrong units and the lower to above 500 units. Ejaculates in cases of aspermia give comparable high results.

Semen is characteristic in that it gives these high values, while other body fluids give values of much less than 5 King-Armstrong units per c.c. The enzyme is resistant to all known body fluids including vaginal secretion; it might, however, be inhibited by vaginal disinfectants and contraceptives. Seminal fluid aged two weeks in a condom-type contraceptive showed no appreciable loss of acid phosphatase activity. Known seminal stains proved to be strongly positive even after many months, six or more.

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*Microbiol. Scan. Suppl. 58: 1, 1946.

**Kaye, S.: Toxicologist to the Chief Medical Examiner, State of Virginia, Richmond, Va.

[7] Weisman, Abner I.: Spermatozoa and Sterility, p. 214, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

[8] Villamil, P.: Crón. méd., Valencia 38: 617, 1934. Reviewed in Sudamer. Endocrin. Immunol. Quimioterap. 18: 184, 1934.

[9] Weisman, Abner I.: Spermatozoa and Sterility, p. 215, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers.

[10] Ito, T.: Deutsch. Ztschr. f. d. ges. gerichtl. Med. 9: 726, 1927.

[11] Simonin, C.: Ann. de méd. lég. 9: 60, 1929.

[12] Husson, A.: Rev. Internat. Criminalist 6: 407, 1934. Ber. ü. d. ges. Physiol. exper. Pharmakol. 86: 390, 1934.

The materials listed in Table 139 contain less than 5 King-Armstrong units of acid phosphatase per c.c. of concentrated pure substance, and in the cases of vaginal fluids, perspiration, feces, nasal discharge, and pus, per unit square centimeter of saturated control cloth.[1]

TABLE 139

BODY FLUIDS	FOOD STAINS	
Vaginal fluids	Tea	Meat gravy
Urine	Coffee	French dressing
Blood (serum)*	Egg white	Beet juice
Blood (whole)	Egg yolk	Custard
Blood (menstrual)	Vegetable juice (V-8)	Starch
Saliva	Orange juice	Mayonnaise
Perspiration	Prune juice	Chop suey
Pus	Beer	Soapy water (Ivory)
Nasal discharge	Milk	Onions
Gastric juice	Cream	Albumin
Feces	Mushrooms	Choline
Rectal smears	Corn	
	Alkaloids, such as quinine, atropine, procaine	

*In carcinoma of the prostate with metastases, this value may be appreciably increased.

Walker Acid Phosphatase Test for Identification of Seminal Fluid[2]

Principle.—

The portion of the garment to be tested is immersed in a bath at pH 5.0 containing alpha-naphthyl phosphate as a substrate, and a suitable diazonium salt. The acid phosphatase of the semen acts on the phosphate ester to liberate alpha-naphthol. This immediately reacts with the diazonium salt to form a brilliant orange-red azo pigment, which is insoluble under the conditions of the test, and which becomes adherent to the background at the site of acid phosphatase activity.

Substrate-Reagent Bath.—

Dissolve 23 gm. sodium chloride
0.5 c.c. glacial acetic acid
2.0 gm. sodium acetate trihydrate
in 90 c.c. distilled water.

Add 1 c.c. of 1% aerosol containing a suspension of 30 mg. anthraquinone-1-diazonium chloride (III)* and 50 mg. of calcium-1-naphthyl phosphate.*

Dissolve the aerosol in warm water without shaking.

Preparation of Anthraquinone-1-Diazonium Chloride (III).*—

Dissolve 10 gm. 1-amine anthraquinone, Eastman technical grade, in 85% sulphuric acid and pour into a large volume of water. Collect the precipitate with suction and wash free from acid with water.

Treat the wet product with 20 c.c. concentrated hydrochloric acid to form a grayish-white crystalline sludge.

Diazotize at 30° to 40° C. by running an equivalent of sodium nitrite, 3.2 gm. in 50 c.c. water, under the surface, with stirring.

Pour the reaction mixture into 10 times its volume of water at 70° to 80° C.

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*These can be purchased from Dajac Laboratories, 511 Lancaster, Leominster, Mass.

[1] Kaye, Sidney, Office of the Chief Medical Examiner, Commonwealth of Virginia, and the Department of Legal Medicine, Medical College of Virginia.

[2] Walker, Joseph T.: New England J. Med. 242: 110, 1950. Presented before the American Academy of Forsenic Sciences, Chicago, Jan., 1950.

Add 0.5 gm. sodium nitrite.

Filter rapidly.

Add sodium chloride to salt out the diazonium chloride in shiny, yellow platelets.

Collect the crystals, wash with cold water, alcohol, and ether, and dry.

Protect from light and store at room temperature, or in a refrigerator. Refrigerated they are stable at least 18 months.

The diazonium compound is available commercially as "Naphthanil Diazo Red AL." It contains 5% zinc chloride and 12% aluminum sulphate. The metal ions completely inhibit acid phosphatase present in the cell nuclei of liver and kidney, whereas the cytoplasmic acid phosphatase is altered slightly. This finding is suggestive of a third variety of acid phosphatase present in cell nuclei.

Preparation of Calcium Alpha- or Beta-Naphthyl Phosphate.*—

Dissolve 14.4 gm. of alpha- or beta-naphthol
in 15.0 c.c. of dry pyridine.*
Dissolve 15.3 gm. phosphorus oxychloride solution
in 10.0 c.c. dry pyridine

Add the phosphorus oxychloride solution drop by drop, or the entire material will be lost.

Add the naphthol solution in portions to the phosphorus oxychloride solution. Heat is developed and a white precipitate separates as a sludge.

Allow to stand at room temperature for 10 minutes.

Cool and add in portions to a solution of 30 gm. of calcium acetate in 200 c.c. distilled water and 100 gm. of ice.

An oily material separates, which slowly dissolves and comes out as a white precipitate.

Add 150 c.c. alcohol.

Collect the white precipitate with the aid of suction.

Wash with 95% alcohol and ether, and dry.

Technic.—

Immerse the garment to be tested in a substrate reagent bath. If acid phosphatase is present, a brilliant orange-red pigment will appear and adhere to the background at the site of acid phosphatase activity. The phosphatase activity of a fresh, dry seminal stain is so intense that within 30 seconds a distinctive, visible coloration occurs, and within 2 minutes the test is complete.

When the semen is mixed with blood, as occurs in rape, an interesting color change occurs. The blood turns to a dirty brownish-gray when immersed in the substrate bath. Wherever semen is present, this color is gradually replaced by the orange red of the phosphatase-catalyzed pigment. Bloody stains should be developed longer than others, so that the pigment formed by the semen may be visible above the background.

If the suspected stain occurs on an unfavorable background, such as a red wool dress, the red pigment of the developed test is not clearly visible. In this case, and in any case in which it is not desirable to apply the reagents directly to the specimen, a portion of the phosphatase of the stain may be transferred to a white filter paper, and its activity detected thereon.

Moisten the filter paper, press it firmly against the suspected area for several minutes, and then immerse the paper in the substrate-reagent bath. Some of the acid phosphatase of the stain on the garment will have diffused into the filter paper sufficiently to cause a print that can be developed.

If desired, place a small portion of the suspected material on dry filter paper. Moisten with a drop of water. Development of the paper will show a red rosette if semen is present.

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*Reagent pyridine contains enough water to decompose the phosphorus oxychloride extensively. Dry by refluxing with anhydrous barium carbonate for several days, distill, and store with sodium hydroxide pellets.

Even if the fabric is tested directly with the substrate-reagent mixture, the procedure does not interfere with the subsequent microscopic search for spermatozoa. The seminal fluid takes on a peach-pink color, but the spermatozoa are not colored, for they do not contain sufficient acid phosphatase. If the specimen is then stained with carbolfuchsin, the spermatozoa stain with the dye. The test serves the double function of demonstrating high acid phosphatase activity and at the same time indicating where a search for spermatozoa is likely to be successful. The phosphatase activity is largely concentrated at the periphery of the stain. Spermatozoa are more plentiful in the central area, where they may be demonstrated microscopically.

Precipitin Test for Identification of Human Seminal Stains

Precipitin tests for the differentiation of semen of human and animal origin can be made by carrying out the test described below.

In the examination of stains in cases of suspected rape, finding spermatozoa is by far the most conclusive evidence of the presence of semen. The procedure described above for the identification of seminal fluid, however, is of value in proof of the presence of semen especially in cases of aspermia or in those cases where complete spermatozoa cannot be identified (twisted tails, etc.).

The injection of rabbits with the semen of man, swine, bull, or horse induces the formation of specific precipitins for the homologous fluid.* In most cases, precipitins develop also for the homologous blood serum, as already known. Extracts of human spermatozoa in salt solution appear to give the same result as the seminal fluid. Seminal precipitin reaction reveals the presence in semen of a substance or substances peculiar to it, and consequently the reaction may be of value in studying the nature of suspected spots and stains.

Precipitin for identification of human semen is made by injecting rabbits intramuscularly four or five times at intervals of three to four days with increasing quantities of human seminal fluid. The first injection is 2 c.c., the next 4 c.c., each successive injection being 2 c.c. more than the preceding one. Six or eight days after the last injection, the animals are bled (see removal of blood from carotid artery, page 2040), and the blood collected in sterile 50 c.c. Rockefeller tubes with cork stoppers. After the blood has clotted, the clot is shaken loose, and the tube is centrifuged at 1,500 revolutions a minute for 20 minutes to separate the serum. The serum is removed with all sterile precautions and placed in another sterile test tube. Serum precipitates are removed from the rabbit's blood serum by mixing it with equal parts of a 1:200 dilution of human blood serum in saline. Allow the mixture to stand at room temperature for one hour; then place in a refrigerator overnight. Centrifuge it at high speed for sufficient time to sediment the precipitate formed from the mixture of the rabbit and human serums. Use the supernatant serum for precipitin tests to identify seminal fluid stains.

Technic.—

Extract the suspected seminal stain in physiologic saline for four to eight hours, using very small amounts of saline. Concentrate the extract slightly, and then centrifuge to remove any sediment that may be present. Remove the supernatant fluid, placing it in a clean, dry test tube. Dilute with physiologic saline until, upon shaking, the amount of foam indicates a 1:1,000 dilution. Be sure the diluted extract is neutral.

Place 0.1 c.c. of antisemen precipitin serum in the bottom of a small heavy walled capillary tube. Allow the extract of suspected seminal fluid stain to run down the side of the tube so that it forms a layer above the serum but does not mix with it. Allow to stand at room temperature for one hour, observing the reaction at 10- to 15-minute intervals. At the same time run a control test using an extract of an unstained portion of the cloth on which the seminal stain was found. A positive reaction is indicated by a white precipitate at the point of contact between the two fluids. This reaction is like the reaction in the precipitin test for the identification of human blood (pages 1162 ff.)

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*Farnum, C. G.: J. A. M. A. 37: 1721, Dec. 28, 1901; Tr. Chicago Path. Soc. 5: 31, 1901; Strube, G.: Deutsche med. Wchnschr. 28: 425, 1902. Pfeiffer, H.: Wien. med. Wchnschr. 18: 637, 1905; Dervieux, M.: Compt. rend. Acad. d. sc. 172: 1384, 1921. Hektoen, Ludvig: J. A. M. A. 78: 704, 1922. Hektoen, Ludvig, and Manly, Leonard, S.: J. Infect. Dis. 32: 167, 1923.

Determination of Blood Group or Type of Semen, Saliva, and Other Body Fluids by Agglutinin Inhibitory Methods

For individual identification of seminal stain the agglutinin inhibitory test is suggested. The results of this test are limited just as the agglutinin absorption test of blood stains. Landsteiner and Levine[1] and Yamakami[2] in 1926 demonstrated that spermatozoa have the power of specifically absorbing group agglutinins from blood serum just as human erythrocytes do. Thus semen from a Group A individual will absorb anti-A isoagglutinins from anti-A serum, and semen from Group B individuals will absorb anti-B isoagglutinins from anti-B serum, etc. This fact at once suggests that it may be possible to trace seminal stains to persons of certain blood group.

The agglutinin inhibitory test below is the method of Wiener.*

Agglutinin Inhibitory Test for grouping stains of semen, saliva, and other body fluids. Test tube method. This test is to be used for secretions only, and may not be used for blood.

Six small Kahn tubes are needed. Each tube must be marked specifically with proper identification. None of the materials used in this test should contain blood or body fluids. It is better to use entirely new glassware for the entire procedure. An extract must be made of the stain, using as small a quantity of physiologic saline as possible. Refer to pages 1169 ff.

In each tube, use 1 drop of the extracted stain or unstained portion with 1 drop of the antiserum. See Table 140.

TABLE 140

TUBES	1	2	3	4	5	6
1 drop of extract of:	Stain	Unstained portion	Stain	Unstained portion	Stain	Unstained portion
Add 1 drop	Anti-A serum	Anti-A serum	Anti-A serum	Anti-A serum	Anti-B serum	Anti-B serum
Allow to stand at room temperature for 1 hour.						
Add 1 drop of	A_1 cell suspension	A_1 cell suspension	A_2 cell suspension	A_2 cell suspension	B cell suspension	B cell suspension

Allow to remain in contact for 30 minutes at room temperature, shaking every five minutes.

Observe macroscopically for agglutination or lack of agglutination.

Confirm by placing a small amount from each tube on a properly labeled glass slide and examine under a microscope.

Cork the tubes and place in the refrigerator for 24 hours and read again.

Tubes 2, 4, and 6 are controls and should be *positive,* that is, there should be agglutination (antiserum against specific cells).

If the results in tubes 1 and 3 are negative, the stain contains agglutinogen A_1 (which removes anti-A_1 and anti-A_2 from anti-A serum).

If the result in tube 3 is negative, the stain contains A_2.

If the result in tube 5 is negative, the stain contains B.

If all the tubes are positive, the stain contains no agglutinogens, and therefore was derived from group O, or there was no inhibition. If both A and B agglutinogens are present, the stain was derived from a group AB person.

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*Personal instruction.

[1] Landsteiner and Levine: J. Immunol. 12: 415, 1926.

[2] Yamakami, K.: J. Immunol. 12: 185, 1926.

This same test may be made on seminal fluid, saliva, urine, tears, sweat, etc. Some individuals are *nonsecretors.* Specimens derived from such persons will fail in the inhibitory experiment, and the secretion cannot be identified.

Explanation.—Isoagglutinogens present in secretions will absorb their corresponding isoagglutinins from known sera. Thus "A" isoagglutinogen will absorb anti-A isoagglutinin, leaving this serum inactive against A cells which are later added. However, if the stain does not contain the "A" isoagglutinogen, the anti-A serum will agglutinate the A cells which are later added. In the case of the subgroups, A_1 might remove both the anti-A_1 and the anti-A_2 agglutinating antibody from anti-A serum, while the A_2 isoagglutinogen will remove only the anti-A_2 isoagglutinin. Thus anti-A serum treated with a blood stain containing A_2 cells will no longer agglutinate A_2 cells but might agglutinate A_1 cells.

"B" isoagglutinogen will absorb anti-B isoagglutinin, leaving this serum inactive against the B cells which are added later.

If the test shows that anti-A serum no longer agglutinates A cells, and anti-B serum no longer agglutinates B cells, then obviously both isoagglutinogens were present and the stain was derived from a group AB individual.

With this information, we may compile Table 141.

TABLE 141.—RESULTS OF INHIBITORY TESTS TO DETERMINE GROUP OF SEMINAL STAINS, ETC.

TUBES						
1	2	3	4	5	6	CONCLUSIONS
	+		+		+	Controls are correct
+	+	+	+	+	+	Stain derived from group O, or inhibition failed to occur
−	+	−	+	+	+	A_1 agglutinogen present
+	+	−	+	+	+	A_2 agglutinogen present
+	+	+	+	−	+	B agglutinogen present
−	+	−	+	−	+	A_1 and B agglutinogens present
+	+	−	+	−	+	A_2 and B agglutinogens present

EVIDENCE IN SUSPECTED SEXUAL ASSAULT

In rape of a child or virgin, there is usually ample evidence in the form of hemorrhage and laceration of tissue. Further evidence may be obtained by looking for traces of semen either on the external portions or on the vagina. The length of time that spermatozoa may be found in the vagina after an alleged assault is varied, some reporting that spermatozoa are found as long as two days after intercourse. While this is true in rape cases without death from the assault, in those cases where death has occurred, spermatozoa apparently may not live very long in the vagina.

In these assault cases, spermatozoa may be found on the clothing by one or more of the tests already described. In addition to the examination of the victim and her clothing, the clothing should be further examined for fibers of the assailant's apparel. We should also examine the clothing of the victim and suspected persons for evidence such as grass, fibers, soil, etc., which may be related to the locality where the crime was committed. In many cases there will be found scratches and skin changes due to the fingernails of the assailant. Therefore, the suspected person should be carefully examined, particularly for skin, hair, and cosmetics, which may be identified as coming from the victim. The scrapings of the nails of the victim should also be examined.

The technic of examination of the victim's vagina for spermatozoa is as follows: Normal salt solution should be gently sprayed over the vagina and perineum of the victim, collected in a clean dish placed under the patient, and then examined. The vaginal wall should be gently washed with a small amount of salt solution injected into the vagina with a clean glass syringe and rubber bulb such as is used in blood cell washing in serologic work. The contents of the vagina should be withdrawn with the same syringe or pipette and, after centrifugalization, examined microscopically. The suspect should also be washed with salt solution sprayed over the entire penis and the collection washed, centrifuged, and examined microscopically.

DEFICIENCY DISEASES

The deficiency of vitamins is responsible for most of the diseases which we call deficiency diseases, but, at the same time, it must not be forgotten that amino acids and minerals and certain fats are also necessary to maintain health; and their absence, as well as the absence of vitamins, constitutes the entire picture of certain deficiency diseases. These latter substances are alluded to as accessory food factors.

Advances in the field of vitamins have come very rapidly. Vitamins have been divided for convenience into the fat-solubles and the water-solubles. The water-soluble ones may be subdivided into ascorbic acid and the B group and those outside of that still expanding list. Spies and Stone[1] have given us their viewpoint on this question. They call attention to the necessity of dissecting the symptom due to depletion of a single factor from the confusing mixture of symptoms characterizing the naturally occurring multiple deficiencies. To that end, studies were made upon human beings, so that many new data have been accumulated on the manifestations of an isolated acute deficiency, more so than on the chronic partial deficiency. These authors alluded to the fact that there are at least twelve identified compounds and mavericks still uncounted herded together under the unwieldly but encompassing name of vitamins. Some of the B complex vitamins function as parts of enzyme systems and thus are essential to the normal respiration of tissues. Other vitamins appear to have a more localized or particularized part to play and, as a group, are concerned with structural integrity more than energy utilization. Spies states that prior to the artificial synthesis of ascorbic acid and thiamine, controlled clinical studies led him and his co-workers to the conclusion that persons with any nutritional deficiency disease had deficiencies of not one but several nutrients. From this, they developed the concept of nutritive failure and so used this concept as a working hypothesis. Nutritive failure, as he uses the term, may be defined as follows: *nutritive failure* is a clinical term which means different things to different physicians. To Spies and his co-workers it designates that stage of malnutrition when the protective mechanisms within the body can no longer compensate for a deficiency of the nutrients and vague symptoms begin to appear. These symptoms arise from a derangement in the tissues as a result of deprivation of nutrients, and they vary greatly from patient to patient. Eventually the signs seen by the discerning physician are interpreted as pellagrous dermatitis or glossitis, beriberi neuritis or beriberi heart disease, hemorrhages or gingivitis of scurvy, cheilosis or the ocular symptoms of riboflavin deficiency. These characteristic symptoms make it possible for the physician to make an accurate diagnosis of a specific deficiency state and to realize that they occur as a response of the body tissues to the deprivation of certain specific chemical substances in food. Thiamine has been considered by some as a cure for nutritional nerve disorders, vitamin A as a cure for night blindness, vitamin C as a cure for scurvy, and niacine as a cure for pellagra. These workers long ago learned to treat the patient for nutritive failure rather than for only the prominent presenting symptoms of a single vitamin deficiency. Nutritive failure is a much more inclusive term than pellagra, beriberi, scurvy, or riboflavin deficiency in that it connotes varying degrees of a mixture of nutritional deficiencies operating simultaneously. It does not indicate why the nutrition has failed but simply that it has failed. The patient, not one disease, must be treated if he is to be completely rehabilitated.

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[1] Spies, T. D., and Stone, R. E.: South. M. J. 40: 46-55, 1947.

One of the most interesting features of the contribution of Spies and Stone is their discussion concerning folic acid. It has been found to play an important role in correcting some macrocytic anemias associated with nutritive failure. A search for a potent antianemic substance of known chemical structrue had been carried on for many years but without success. Niacine, riboflavin, thiamine, pantothenic acid, pyridoxine, choline, inositol, para-aminobenzoic acid, and uracil, singly and combined, administered orally and intravenously, showed no indication of having antianemic properties in human beings. Many others were tried without showing any antianemic effects. Vitamin B_{12} has been used successfully in the treatment of pernicious anemia. Refer to page 720.

The *Lactobacillus casei* factor, commonly called folic acid, seems to fill a long-searched-for want. It has the appropriate chemical term ''pteroylglutamic acid.''

Spies and his associates have found a favorable therapeutic response to synthetic folic acid in the cases shown in Table 142.

TABLE 142

TYPE OF ANEMIA	NUMBER OF CASES
Addisonian pernicious anemia*	78
Tropical sprue	76
Nontropical sprue	2
Nutritional macrocytic anemia	46
Macrocytic anemia of pregnancy	3
Nutritional leukopenia	8
Cirrhosis of the liver with macrocytic anemia (7 cases did not respond)	5

*Refer to remarks in text below.

This favorable therapeutic response can be detected in the bone marrow soon after therapy is initiated. The numbers of megaloblasts and early erythroblasts decrease progressively and the late erythroblasts and normoblasts increase. Eventually the normal ratio of nucleated red blood cells and white blood cells of the marrow is re-established. Reticulocytosis can be detected in the bone marrow sometimes as early as the second day. Its beginning is frequently detected in the peripheral blood from about the third to the fifth day of therapy. A peak is reached on the sixth to tenth day. The height of the rise varies from case to case depending upon the severity of the anemia, the adequacy of the dose, and the presence or absence of complications. In addition to the reticulocytosis there is a gradual increase in the number of red blood cells and in the hemoglobin. When thrombocytopenia and leukopenia are present, increases toward normal are to be expected.

Spies and his co-workers suggested that folic acid will maintain the increased red blood cell and hemoglobin levels which have been produced either by folic acid or by liver extract in most cases and that it may control sprue more effectively than does liver extract. There is no obvious explanation for the decrease in erythrocytes and in hemoglobin which followed remission in two of the three patients who were in relapse at the time folic acid therapy was initiated by them. Spies suggested that the folic acid caused depletion of some other nutrient essential for erythrocyte maturation. Furthermore, *folic acid does not always prevent the occurrence of combined system disease nor does it reverse the degenerative process once it has begun.* They believe that folic acid works as a part of an enzyme system and as yet they have observed nothing which led them to change their point of view. It will not protect against the neural disturbances which frequently develop in pernicious anemia. One of the most significant features of their observations is this question which they presented at the conclusion of their last paper[2]—since folic acid conjugates are present in food and are probably stored in our bodies and since the normal person and persons with nutritional macrocytic anemia and the macrocytic anemia of sprue, in contrast to persons with pernicious anemia, seem able to liberate the *L. casei* factor, is it not likely that liver extract and substances that act similarly may contain a substance capable of liberating from the tissues the substances which in turn act on the cellular components in the bone marrow? Could it not be that the ineffective utilization of the naturally occurring conjugate of folic acid by patients with addisonian pernicious anemia is secondary to a deep-seated biochemical defect?

Vitamin B_{12} is discussed on page 720.

Vitamin A Deficiency

Deficiency of vitamin A leads to changes referable to ocular disturbance. Night blindness is a definite example of vitamin A deficiency. Xerosis, xerophthalmia, and keratomalacia are severe stages of the same disease. Vitamin A belongs to the group of their higher alcohols. It is formed by the metabolic activity of the animal organism from vitamin substances produced by plants, or carotenes, carotenoids, or pigments. Four of these carotenes may be converted into vitamin A; i.e., β-carotene, α-carotene, carotene, and cryptoxanthin.

Vitamin B Deficiency

Thiamine or Vitamin B_1 Deficiency.—B_1 deficiency leads to beriberi.

Beriberi

Beriberi is a food deficiency disease due to the absence from the dietary of a neuritis-preventing vitamin, B_1 (thiamine). It is found in the Orient where the diet is preponderatingly one of rice. The grain used there is polished rice, in which the vitamin-containing layers are rubbed off. The neuritis which occurs in beriberi involves not only the nerves of the extremities, but also the pneumogastric nerve. It is the manifestation of cardiac disturbances which differentiates this form of neuritis from the neuritis due to alcohol or arsenic.

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[2] Loc. cit.

The disease was described in 1642 by Bontius, although the Chinese writers of the seventh century referred to it. It is found in Japan, Netherlands East Indies, and the Eastern Coast of Brazil, in India, Indo China, Malay Peninsula, Eastern China, Philippine Islands, East and West Coasts of Africa. More than three hundred cases occurred among the British soldiers in Mesopotamia during World War I.

The history of the connection between beriberi and vitamin B_1 dates from 1884 to 1941.* Takaki, Director-General of the Medical Service of the Japanese Navy, was one of the first to note the relationship of beriberi to a deficient dietary. By substituting other foodstuffs for a portion of the rice which had previously constituted a substantial part of the diet, he succeeded in virtually eradicating beriberi from the Japanese Navy as early as 1884. In 1897, Eijkman in Java demonstrated that a condition closely resembling beriberi could be produced in fowls by a diet consisting exclusively of polished rice. His work is well known, especially since his discovery occurred paradoxically quite by accident during the course of experiments designed to prove the infectious nature of the disease. His successor, Grijns, fully recognized the significance of his discovery, but it was not until Fletcher in 1905 and Fraser and Stanton in 1909 reported their definitive results in human beings that the dietary origin of beriberi became accepted to any great extent beyond the confines of the Netherlands East Indies. The next contribution occurred in 1911, when Funk succeeded in extracting from rice polishings a crystalline fraction of material which proved to be effective in curing experimentally induced polyneuritis in pigeons.

Funk advanced the deficiency theory as the cause not only of beriberi but also of scurvy, rickets, and pellagra, and proposed the term "vitamine" as a general designation of the accessory food factors which he considered essential for the prevention of these diseases. During this time, Vedder had been active in studying the problem in the Philippines, and it was there that he introduced Williams to the subject in 1910. Williams in the Philippines cured many cases of infantile and adult beriberi with potent extracts from rice polishings. It was in 1916 that Seidell introduced fuller's earth as an adsorbent to obtain a concentrated fraction. This contributed measurably to the ultimate isolation of the vitamin, as well as to the differentiation and isolation of other members of the vitamin B complex. In 1926, Jansen and Donath, working in the same laboratory in Java where Eijkman had pioneered thirty years earlier, isolated from rice polishings a crystalline substance which showed marked antineuritic activity. They used adsorption on acid clay, elution with baryta, and fractional precipitation with silver nitrate. They used rice birds for the bioassays because these small birds contract avian polyneuritis within ten to fourteen days when fed on an exclusive diet of polished rice. In 1932, Ohdake obtained a crystalline product which was far less potent than that of Jansen and Donath, but his formula was more accurate. In 1935, Williams and his associates determined the construction of the crystalline substance. It was in this year that large-scale production was undertaken with the cooperation of Merck & Co., Inc., and it became possible to conduct extensive analytical investigations on the crystalline material. Williams and his associates split the vitamin B_1 molecule into progressively smaller pieces until they obtained cleavage products whose identities could be determined. They then reconstructed the molecule from these organic compounds of definite structure to form a product identical with the vitamin as obtained from natural sources. These analytical investigations were facilitated by the knowledge, gained from earlier experience, that the vitamin is quantitatively split into two components by neutral sulphite solution at room temperature. By 1936, Williams was able to advance a revised formula which accurately denoted the molecular structure of the crystalline substance. In the next year Williams and his associates succeeded in synthesizing the compound. This synthesis was described by Cline, Williams, and Finkelstein in a paper emanating from the Merck Research Laboratories. As a result of the synthesis of thiamine hydrochloride by Williams and his associates, we now have an important therapeutic agent, identical in every respect with the naturally occurring vitamin.

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*Historical facts from publication by Merck & Co., Inc., Rahway, N. J.

The disease is described under two types: (1) wet or dropsical beriberi, in which the victims are affected with general edema; (2) dry or atrophic type in which the absence of beriberi-preventing vitamins constitutes the leading features. In death from wet beriberi, the tissues are very moist. There are generally present pleural and pericardial effusions. The lower end of the stomach and the upper portion of the small intestines, in particular the duodenum, show marked congestion with hemorrhagic areas. The heart chambers show a dilatation with hypertrophy. The heart muscle has the faded-leaf appearance indicative of fatty degeneration, with microscopical segmentation and vacuolation of the fibers. Lungs show congestion and at times present the characteristics of pulmonary edema. The kidneys are congested. There is back pressure in the right heart with dilatation of the central vein of the liver lobules which at times is productive of atrophy of the adjacent liver cells. The muscles supplied by the affected peripheral nerves show more or less atrophy according to the duration of the disease. The peripheral nerves show nerve degenerations varying from slight medullary degeneration in a few fibers to a complete destruction of the nerve, giving a Wallerian degeneration; that is, vacuolation and formation of myelin droplets in the medullary sheath with fragmentation of the axis cylinder. The key to beriberi is the peculiar and striking selection of the vagus nerve in the degenerative processes as well as those of the peripheral nerves of the extremities. This vagal involvement produces disturbances of the heart particularly, and lungs in lesser degree, and thus differentiates beriberi from other forms of multiple neuritis.

The most common symptoms are those connected with degenerations involving the peripheral nerves of the extremities. The motor nerves are more involved than the sensory ones, there being rarely complete anesthesia, but rather a blunting sensation as though a piece of cloth were interposed between the examining instrument and the skin. There is weakening of the muscle power as shown by the grip of the hand or weakness of foot muscles. In more advanced cases we may observe foot- and wristdrop. The unsteadiness of gait is not true ataxia, as the patient does not clearly show the Romberg sign.

It is muscular weakness rather than incoordination. The gait is the "steppage" one of peripheral neuritis, the patient walking as though extracting one foot after the other from clinging mud. Later on, the gait becomes a shuffling one. The mind is clear. The knee jerk is absent. The extensors of arms and legs are more markedly affected than the flexors. Diagnosis is made by the edema, the cardiac involvement, and signs of peripheral neuritis.

There are certain types of neuritis which are vitamin B_1 deficiencies, such as polyneuritis associated with infections and disturbances associated with chronic alcoholism. Polyneuritis of pregnancy has been shown to be due to vitamin B_1 deficiency.

Vitamin B_2 Complex Deficiency

Vitamin B complex has been split into at least six fractions, of which three have been isolated, synthesized, and may be purchased, namely, riboflavin, niacin (nicotinic acid), and vitamin B_6. Vedder[1] has discussed the relationship of sprue to vitamin deficiency. In sprue, there is definite diarrhea. Vitamin B_1 is poorly absorbed in any case of diarrhea and one of the first symptoms of vitamin B_1 deficiency in practically all experimental animals is a diminished appetite and loss of motility of the digestive tract. Vedder believes that symptoms of sprue are delayed by the administration of B_2 complex either by mouth or parenterally.

Regarding vitamin D and sprue, this vitamin is fat soluble so that there must be failure of absorption in any case having steatorrhea. This aggravates the condition in cases of nontropical sprue.

Discussing vitamin K in connection with sprue, Vedder calls attention to the fact that this is a substitution product of naphthoquinone and is fat soluble. A sufficient supply must be afforded or prothrombin cannot be formed in the liver in adequate amount and this results in a failure of the blood to clot properly. It is poorly absorbed when steatorrhea exists because it is a fat-soluble vitamin. Snell states that a hemorrhagic diathesis has been described in connection with tropical sprue. In one case the normal prothrombin content of the plasma was reduced more than 50 per cent. From these considerations, Vedder concluded that a deficiency state of lesser or greater extent is demonstrable in all cases of sprue depending upon the severity and duration of the case, and that an important part of the symptomatology is caused by deficiency in the B_2 complex. Deficient parathyroid and adrenal secretion have been suggested as the cause of the intestinal dysfunction in sprue.

Sprue.—Refer to page 722.

Riboflavin Deficiency

The lesions of riboflavin deficiency were described by Sebrell and Butler in 1938.[2] These lesions were a cheilosis, a stomatitis at the angle of the mouth, a seborrheic dermatitis, a glossitis, and changes in the vascular supply of the cornea. These lesions are also found in pellagra, in which there is an associated riboflavin deficiency. Laboratory diagnosis of riboflavin deficiency has not progressed to the point where any particular technic may be recommended. However, certain methods of assay have been published. The literature on the chemical methods may be summarized as follows: Chèvremont and Comhaire[2] report that lactoflavin was detected in fixed tissue sections by the red color it develops when partially reduced with Zn and HCl. Further reduction converted it to the leuko compound. The color was restored by mild oxidation with H_2O_2. The injection of the metabolism-accelerating compound cyclopentyldinitrophenol into guinea pigs increased the lactoflavin content of the liver cells. Shaw[3] described a method for the estimation of riboflavin in commercial liver extracts, based upon converting the substance to luminoflavin by exposing a solution in N/2 sodium hydroxide to unfiltered ultraviolet radiation, and comparison of fluorescence with standard luminoflavin preparations. It was also found that riboflavin is not stable to nitric acid as was previously thought.

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[1] Vedder, Edward B.: Am. J. Trop. Med. 20: 345, 1940.

[2] Sebrell, W. H., and Butler, R. E.: U. S. Pub. Health Rep. 53: 2282, 1938.

Sullivan and Norris[4] described a photometer which utilizes a zero-potential circuit in connection with a blocking-layer photocell. This instrument gives a linear response to varying light intensities and offers advantages in regard to proper use of the photocell, reproducibility of results, and application to certain spectrophotometric measurements. Riboflavin may be extracted from dried milk products by refluxing with a dilute solution of acid in 75 per cent acetone. Certain unstable colored impurities may be destroyed by including from 1 to 3 per cent of hydrogen peroxide in the solvent. The resulting solution, after neutralizing and filtering, may be used for quantitative measurements since riboflavin is not preferentially adsorbed by the precipitated milk solids. By reduction with sodium hyposulphite, 90 per cent of the color of riboflavin is removed. Observation of the light absorption before and after reduction enables one to calculate the concentration of the solution to ± 0.05 γ per c.c.

Niacin (Nicotinic Acid) Deficiency

Pellagra is the principal disease which is an expression of niacin deficiency.

Pellagra

This is another food deficiency disease with a possible infectious factor as well. It is a chronic disease with periodic exacerbations. The symptoms consist of mild neurasthenic manifestations in the winter followed in the spring by disturbances of the alimentary tract, consisting of stomatitis, burning sensations going up the esophagus, gastric eructations, and recurring diarrheas. In addition to the neurologic and alimentary tract symptoms, there are the third and most important group, those of the cutaneous system. On the skin is a pellagrous eruption, characterized by strikingly symmetrical, sharply delimited patches of erythema, resembling sunburn. The sites of preference are backs of hands, extending up the forearms, bridge of nose, or neck. The neurasthenia tends to pass into a toxic psychosis or even a confusional insanity.

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[2] Chèvremont, M., and Comhaire, S.: Compt. rend. Soc. de biol. **128**: 1167-1169, 1938.
[3] Shaw, G. E.: Quart. J. Pharm. & Pharmacol. **12**: 541-549, 1939.
[4] Sullivan, R. A., and Norris, L. C.: Indust. & Engin. Chem., Anal. Ed. **11**: 535-540, 1939.

The first description of this disease was by Casal who, in 1735, described the disease as it existed in the Asturias. It was described by other Spanish observers. Casal called the disease *mal de la rosa.* In 1907 pellagra was described in the United States and since then a number of cases have been found here. It has been reported from Italy, Balkan States, Greece, Turkey, Spain, and Portugal. There were about 100,000 cases in 1906. It is widespread in Lower Egypt, where the inhabitants live on millet instead of maize.

The work of Goldberger in respect to establishing the relativity of a deficiency of the pellagra preventive factor (P.P.) is now as generally accepted as is the relation of vitamin B to beriberi. In 1925 Goldberger and Tanner announced the existence of a hitherto unrecognized factor necessary in the diet to prevent or cure pellagra. This P.P. factor is associated with the antineuritic factor which has been synonymous with the term, ''vitamin B.'' Yeast is at present the richest known source of the P.P. factor, as it is of the antineuritic complex. Both are water soluble. Goldberger determined the presence of P.P. factor in certain foods studied by him and his associates by noting the activity of each in preventing pellagra among significant groups of pellagrins, and also by determining the effectiveness of each to prevent blacktongue in dogs. Blacktongue in dogs, according to Goldberger, is the analogue of pellagra in human beings. There are no constant or characteristic pathologic changes in pellagra. In the second stage the urine shows an indicanuria and the feces an abundance of skatol. Gastric juice shows anacidity and deficiency in pepsin. At autopsy we find marked emaciation, with marked degenerative changes in the corium with slight cellular infiltration. There is superficial atrophy in the epidermis with thickening in the stratum granulosum. The spleen shows atrophy. Liver and kidneys often show fatty change. In general, the changes are those of a senile character. These cases show a multiplicity of symptoms. There is a history of more or less prolonged neurasthenic manifestations during the preceding winter, dizziness, insomnia, apprehension, occipital heaviness, and fatigue. Sensitiveness of the mouth and slight epigastric discomfort are noted. With the eruption, there is more marked alimentary tract disorders consisting of stomatitis, gastric disturbances, with a recurring diarrhea. The eruption shows localized, sharply delimited, strikingly symmetrical skin lesions of those parts of the body which are chiefly exposed to the sun's rays. There is a diagnostic triad in a typical case as follows:

1. Symmetrical sharply delimited erythemas of certain portions of the skin exposed to the sun. 2. Alimentary tract disturbances of stomatitis, epigastric and substernal soreness and burning, with pyrosis and a recurring diarrhea. 3. Neurological manifestations in which a prodromal neurasthenia is followed by paraesthesias, in which severe buring sensations are prominent, with alterations of deep reflexes, tremors, and, in more advanced stages, a confusional insanity. Burning sensations are to be noted in the mouth, gullet, and stomach as well as in the skin. Palms of the hands and soles of the feet often give a burning sensation.

Vitamin C Deficiency

The chief example of vitamin C deficiency is the disease known as scurvy, showing anemia, loss in strength, changes in the gums, with swelling and hemorrhages throughout the body. While scurvy has been known for centuries, occurring among sailors, inmates of institutions suffering from poverty, exposure, and lack of proper diet, a clinical form had not been seen until recently when hypovitaminosis C or a latent form of scurvy was recognized by its vague symptoms, inadequate intake of this vitamin, and by laboratory methods.

Vitamin C is ascorbic acid or cevitamic acid.

Vitamin D Deficiency

Vitamin D deficiency is manifested by the symptom complex known as rickets. Vitamin D deficiency causes a disturbance in the metabolism of calcium and phosphorus.

Laboratory Tests.—The determination of phosphate concentration of blood, together with calcium concentration of blood, is a useful test in diagnosis of suspected vitamin D deficiency and rickets. The calcium findings may be normal or increased in children, but when reduced, there is a likelihood of the onset of tetany. There is decreased serum phosphate in rickets, while the serum phosphatase is increased. In normal children, Howland and Kramer* found values between 4.5 and 6.8 mg. per 100 c.c., averaging 5.4 mg., but in rachitic children, the values ranged between 0.6 and 3.2 mg. per 100 c.c., averaging 2.0 mg. The average was thus less than 50 per cent of the normal average value. Howland and Kramer further established an empirical rule regarding the relation between the serum calcium and phosphorus. They showed that if the product of the serum phosphorus and calcium fell below 30, rickets developed, whereas if the product remained above 40, the child escaped this disease.

So far as blood phosphatase is concerned, it is elevated in rickets and is thought to be increased before changes in the phosphorus and x-ray changes appear. Thus it may be of great value in early diagnosis. It remains elevated long after treatment is begun, apparently returning to normal only after healing is complete or nearly complete.

(See pages 447 ff. for method of determining serum inorganic phosphate.)

Vitamin E

This vitamin has been known as an antisterility vitamin. Effect of lack of vitamin E in female rats causes premature death of the fetus. It is barely possible that a corresponding condition in human beings is habitual abortion although this has by no means been proved.

Vitamin K Deficiency

The absence of vitamin K induces hemorrhagic disease of the newborn. Vitamin K affects blood clotting only by its effect on the prothrombin concentration. Hemorrhagic states related to vitamin K deficiency are hemorrhages accompanying obstructive jaundice, hemorrhages in connection with diseases of the gastrointestinal tract such as sprue, celiac disease, ulcerative enterocolitis, and icterus gravis.

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*Howland, J., and Kramer, B.: Am. J. Dis. Child. 22: 105, 1921.

Laboratory tests for vitamin K are concerned with the prothrombin time of blood plasma. (See facts on prothrombin test and liver function, pages 688 ff., 895 ff., 903, 958.)

CHAPTER X

BACTERIOLOGIC APPLICATIONS TO CLINICAL DIAGNOSIS

THIS CHAPTER HAS BEEN REVISED BY OSCAR FELSENFELD, M.D., M.S., PROFESSORIAL LECTURER, UNIVERSITY OF ILLINOIS COLLEGE OF MEDICINE*

GENERAL CONSIDERATIONS

This chapter includes the discussion of means and methods used in medical and sanitary bacteriology for the isolation and identification of disease-causing agents. When work along these lines is carried out, one must realize that bacteriologic technics applied to medical laboratories are regulated by the same rules as in any other field. With the exception of a very few procedures, for example, plating of stools, scrupulous adherence to the rules of sterility is imperative. One cannot produce good results in nonsterile dishes, contaminated plates, and carelessly plugged tubes. Adequate initial plating of the sample is of paramount importance. Only well-isolated colonies may serve for further study, and contamination must be avoided during the attempts to determine the species which produced the colony. It is surprising how these basic rules are often neglected in some medical laboratories. The Reviser of this chapter saw hundreds of plates streaked with three thick strokes of a loop and so thoroughly overgrown that not even repeated restreaks could assure the isolation of the organisms. He was faced with innumerable airborne molds grown on Sabouraud plates into which every technician in the laboratory breathed, every nurse delivering samples coughed, and every maid swept the dust of the floor. He also received, from otherwise excellent laboratories, tubes labeled ''pure cultures'' from each of which he was able to isolate four or five different organisms. One may suspect that the mistrust of some physicians toward certain laboratories regarding bacteriologic reports is based upon the insufficient attention devoted to such examinations.

This chapter, therefore, has been completely rewritten, in order to present up-to-date technics, and to underline those features which are not always matters of common knowledge. On the other hand, limited facilities result in limited reporting. This does not mean, however, that a small laboratory shall declare each gram-negative diplococcus a *Neisseria gonorrhoeae* or disregard every Salmonella because the typing of the latter can be carried out only in large establishments. Most State, Municipal, and Federal Public Health Laboratories will be glad to render advice or practical aid in difficulties encountered during bacteriologic work. Such aid must be freely called upon when working with limited means on stools, anaerobic organisms, tularemic material, viral and rickettsial diseases, atypical cocci, molds and yeasts, etc. Every laboratory worker must set before himself a goal of optimal achievements under the particular circumstances of his laboratory, and send all other cultures and materials to better equipped institutions.

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*At present, Lieutenant Colonel, MC, USAR.

As to the general methods used in bacteriologic work, diagnosis is made by culturing; in some instances and with certain probabilities, by microscopic examination. The general procedure is to examine material taken under sterile conditions, except for stools, throat smears, cough plates, vaginal smears. First a microscopic examination is performed, using careful sterile technic, care being taken not to contaminate the material for further examination; then plates are inoculated. During the subsequent procedure suspicious colonies are "fished" into so-called differentiating media and studied for certain reactions and microscopic structure. In some cases enrichment fluids are used to increase the number of viable organisms, or concentration methods are applied. The final diagnosis is secured by serologic tests, which, unfortunately, are not used enough in many medical laboratories. Animal inoculations are infrequently carried out, because of the great efficiency of in vitro methods. Preliminary and final reports are issued in due time.

Of primary importance, as stated above, is the creation of sterile conditions, in order to avoid contamination. Media satisfactory as to composition, pH, and moisture are also of great importance. Microorganisms, perhaps with the exception only of *Pasteurella tularensis,* definitely refuse to grow on very dry surfaces. When, in addition to the above requirements, a good streak is used, proper transfers are made, and legible slides are produced, reliable reports will result.

Considerable difficulty arises from the unfortuante conception shared by some medical workers that everything isolated from a patient is necessarily pathogenic. The intestines, the vagina, the respiratory system, and all mucosae which have contact with the outside world are full of nonpathogenic microorganisms. Not even hemolysis is an indicator of pathogenicity. Many members of the *Bacillus subtilis* group, several coliforms, etc., will produce wide areas of hemolysis, without always being of importance. In other cases too much faith is placed upon microscopic examination. Not every Streptococcus, not every granulated rod, and not every sporeformer is a cause of disease.

When these basic rules are kept in mind, bacteriologic technic becomes a better means of laboratory diagnosis.

Preparation of Glassware for a Bacteriologic Laboratory

General Rules

All glassware must be cleaned and sterilized before use.

Rinse thoroughly in tap water.

Immerse in a 1:1000 nitric acid solution for one hour.

Rinse thoroughly in tap water to remove the acid.

Rinse in distilled water.

Plug test tubes, cylinders, flasks, etc., with nonabsorbent cotton.

Nonabsorbent cotton is used exclusively for making plugs.

Sterilize in the dry heat sterilizer at 170° C. for two hours.

Petri dishes should be wrapped in paper or put in metal cans before sterilization.

All syringes and needles must be wrapped in paper and sterilized in the dry heat sterilizer at 170° C. for 2 hours.

Slipknots must be made instead of double knots so that packages may be opened easily.

The contents of all packages must be marked, together with date of sterilization, before placing them in the oven.

Syringes and needles can be sterilized in cotton-plugged test tubes.

Pipettes and droppers should have a loose plug of nonabsorbent cotton, and should be wrapped in paper before sterilization. Place an X mark at the mouth end after wrapping. The use of metal cans is also recommended.

Pipettes kept in cans may be wrapped individually in paper, so that they do not become contaminated when the container is opened. If pipettes are kept unwrapped in the cans, the cans should be resterilized by dry heat after each opening. If care is exerted, however, when individual pipettes are taken out, it is satisfactory to resterilize the opened cans only once a day.

Some glass wool should be placed in the bottom of the pipette cans to prevent breakage of the pipette tips.

Slides and cover glasses should be washed in the manner described above, then immersed in a mixture of equal parts of ether and alcohol, then dried with an old linen cloth. The cloth must be free of lint. Or use the method described on page 612.

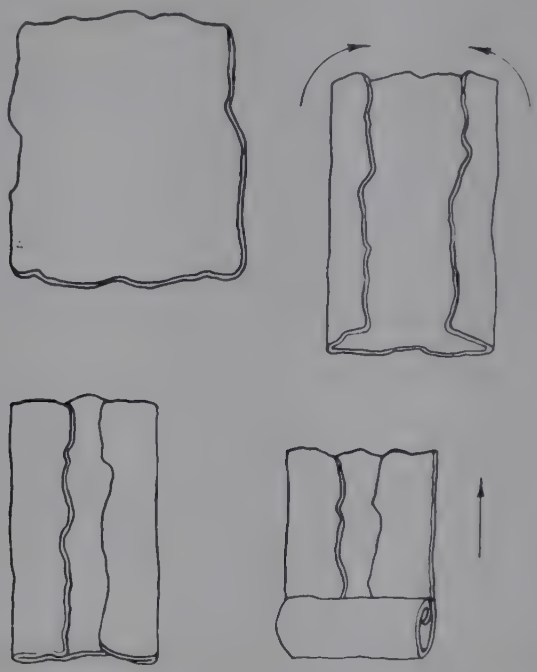

Fig. 323.—Rolling cotton plugs. (Courtesy Dr. Oscar Felsenfeld.)

Sterilizing Tubes and Flasks

Ordinarily test tubes, flasks, etc., in bacteriologic procedures are closed by inserting stoppers made of nonabsorbent cotton plugs. The method of plugging test tubes is to pull off a small piece of cotton, place it over the open end of the tube and thrust it in place by means of a glass rod, pencil, or forceps, so that the edges of the cotton will fit smoothly.

Such plugging can be used only for media stored in a refrigerator and utilized quickly, because it is an insufficient protection against evaporation. In all other cases rolled plugs must be made. An approximately quadrangular piece of nonabsorbent cotton is separated from the roll. Two sides of this piece are folded inward, to about one-fourth of its width. Then the cotton is firmly rolled along the longitudinal axis and the plug inserted by gentle rotation. See Fig. 323.

The inner end of cotton plugs for flasks is protected with one or two layers of gauze or cheese-cloth. A quadrangular piece of the gauze is cut. The inner end of the cotton plug is put in its middle, then the corners lifted and the whole inserted into the flask in such a way that the neck of the flask is not in contact with cotton.

Covering the plug and the neck of bottles with paper and fastening this cover with a string around the neck is helpful.

Some laboratories use rubber fingers to cover the plugs and the necks of flasks.

Paraffin renders excellent results. It is applied to tubes with live cultures to seal the plugs.

A closure has been devised which has been found quite serviceable. It is called the "Quicap" closure and it is especially designed for flasks. (See Fig. 324.) It consists of a moisture-proof sheet of DuPont cellophane which covers the opening of the flask and, when held in place by means of a Quicap collar, forms a tight, hygienic seal. These closures will withstand fifteen pounds of pressure for thirty minutes. To use these caps pick up the cellophane cover from the dispenser and place, with a Quicap collar, on top of the flask opening. Tilt downward and over the flask head. A slight upward pressure on the collar assures a snug fit.*

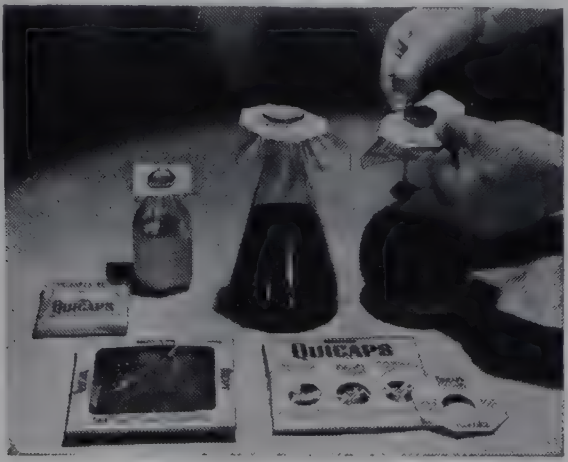

Fig. 324.—Quicap closures for flasks, etc. (Courtesy Quicap Co., Inc., Brooklyn, N. Y.)

Good protection against evaporation is afforded by screw-capped tubes. Snieszko** recommended their use. When media are sterilized in such bottles, the caps are screwed on partially to allow some air to escape. Caps are tightened immediately after the media are taken out of the sterilizer.

A very ingenious method of plugging culture tubes with nonabsorbent cotton is carried out by the use of Coiler cotton.† This is nonabsorbent cotton furnished in long strands. All that is necessary is to pull off a small plug and insert it in the test tube. It fits snugly into the usual test tubes for preparing culture media. (Fig. 325.)

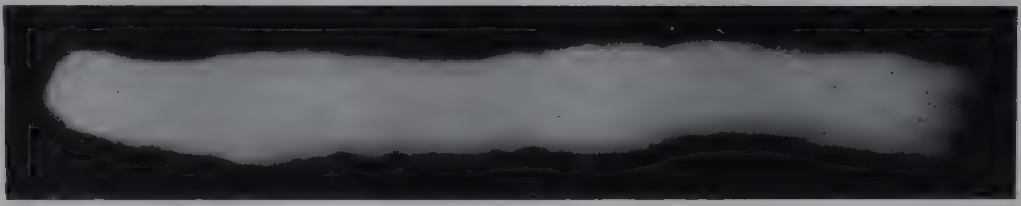

Fig. 325.—"Coiler" nonabsorbent cotton for plugging culture tubes. (H. J. Niehaus and Son, Kirkwood, Mo.)

Empty, plugged test tubes and flasks are sterilized by dry heat at 170° C. for 2 hours. Let the temperature of the oven fall to 100° C. before taking out the glassware, to prevent cracking of nonresistant glass, and to keep from burning the hands.

Sterilizing Glass Syringes

The life of a good, all-glass hypodermic syringe, sterilized under proper conditions, is from 50 to 200 hours boiling, depending upon the quality of glass, fineness of grinding, and fit of the plunger.

B-D Yale and Luer syringes can be repeatedly sterilized by boiling, autoclaving, dry heat, or by suitable chemical agents. If syringes are contaminated with spore-bearing organisms, boiling does not suffice.

Ordinary tap water, not containing an excess of alkali, has been found the best medium for sterilizing syringes by boiling.

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*This equipment is made by Quicap Company, Inc., 233 Broadway, New York.

Science **100: 435, 1944.

†This can be procured from H. J. Niehaus and Son, Agents, P.O. Box 3817, Kirkwood, Mo.

B-D Yale and Luer syringes may be safely placed directly into the hot water; this saves time and hastens sterilization, and without separating barrel and plunger. The syringes, however, must be clean and the plunger pushed all the way down before placing in the sterilizer.

The separation and reassembly of parts has been the cause of much breakage and loss of time.

Should it be necessary to use water which has an excess of alkali, either sterilize the barrel and plunger together, or swab out with warm water, then with alcohol, and dip the end of the plunger in mineral oil and thoroughly lubricate the ground glass surfaces. The oil protects the syringe to some extent against erosion.

Precautions.—Do not boil in water to which bicarbonate of soda or sterilizing tablets containing this chemical have been added as it, in common with all alkalies, attacks glass.

Physicians who regard distilled water as the ideal medium in which to boil their syringes are reminded that distillation removes the silicate for which water has an affinity. When boiling glass in distilled water, a certain amount of silicate in the glass is taken up by the water and a slow process of deterioration takes place.

The routine procedure for bacteriologic use should be dry heat sterilization, at 170° C. for 2 hours, of paper-wrapped syringes, or syringes kept in test tubes.

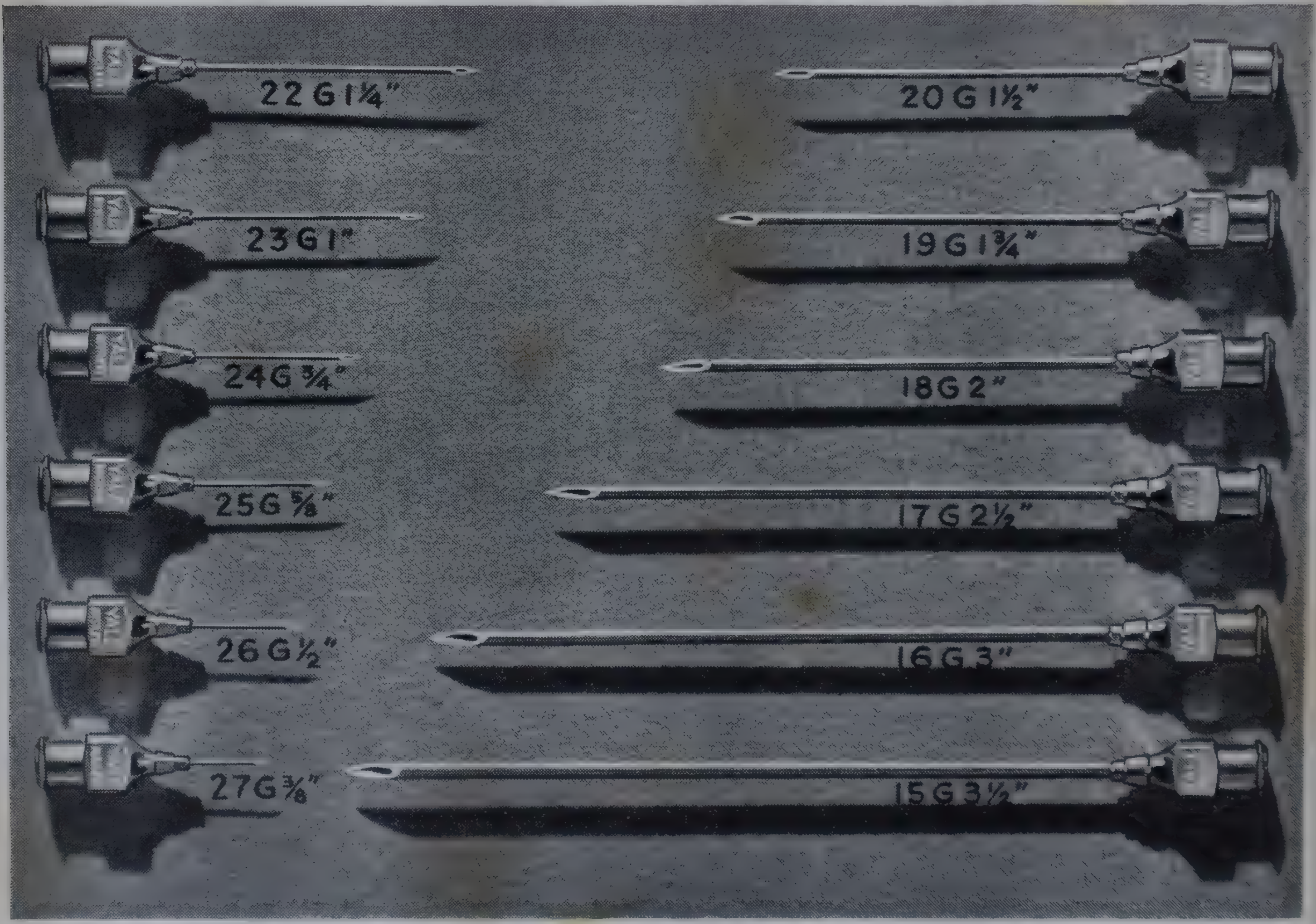

Fig. 326.—Actual size needles showing length and gauge. (Courtesy Becton-Dickinson & Co.)

Sterilizing, Cleaning, and Care of Needles

After use, all needles should be immediately washed and then rinsed with alcohol or ether. This is readily done by attaching the needle to a syringe and drawing alcohol or ether through the needle several times.

Thoroughly dry the bore of the needle with compressed air or by the use of the Brunet-Flagge Needle Drier.

Before putting away carbon steel needles (such as the Yale), dip the needle wire or stylet in oil or liquid petrolatum and insert. This prevents rust and corrosion and greatly increases the life of the needle. Erusto and Medical Center needles should not be oiled.

The points should now be carefully inspected and if blunt or scratchy, sharpened on a fine oilstone.

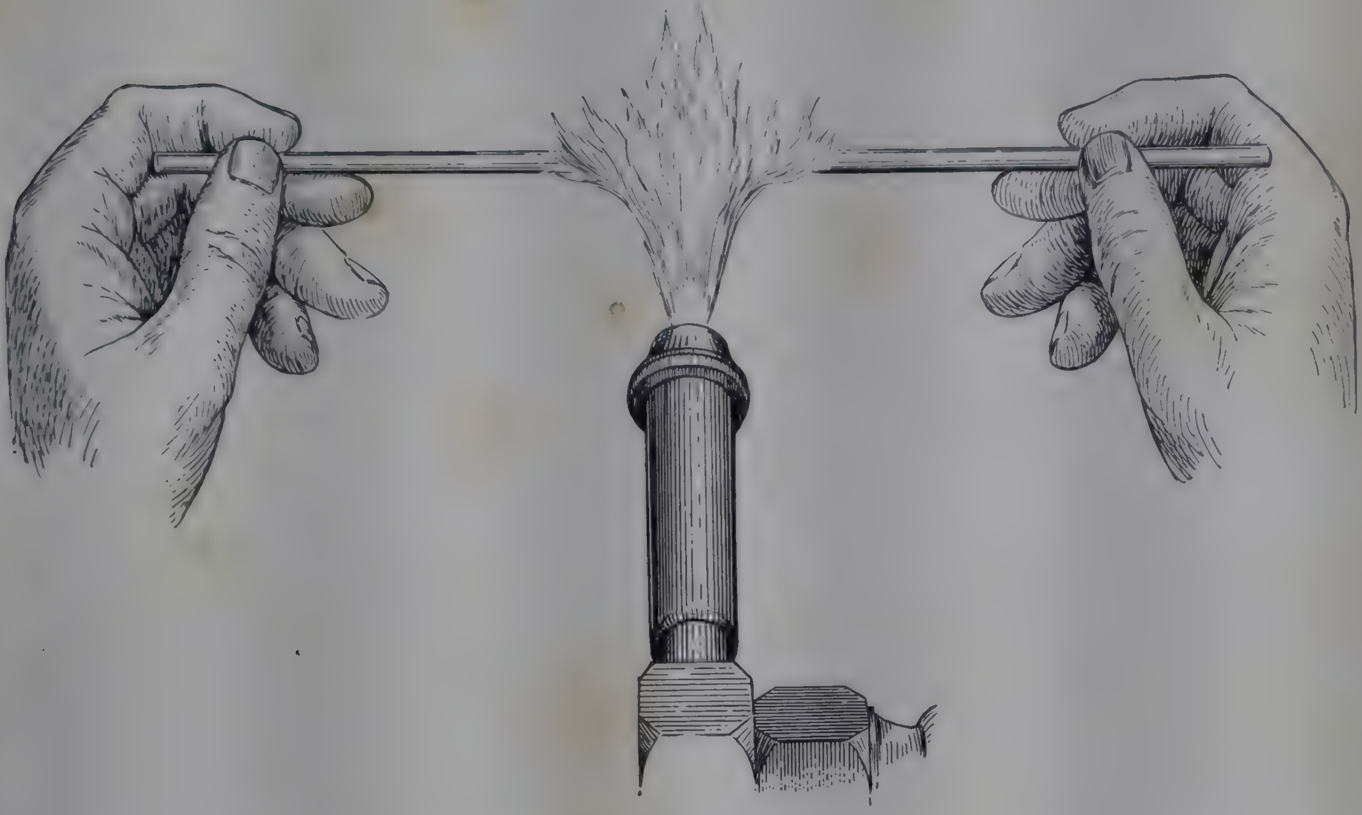

Fig. 327.—Heating glass tubing before drawing a capillary.

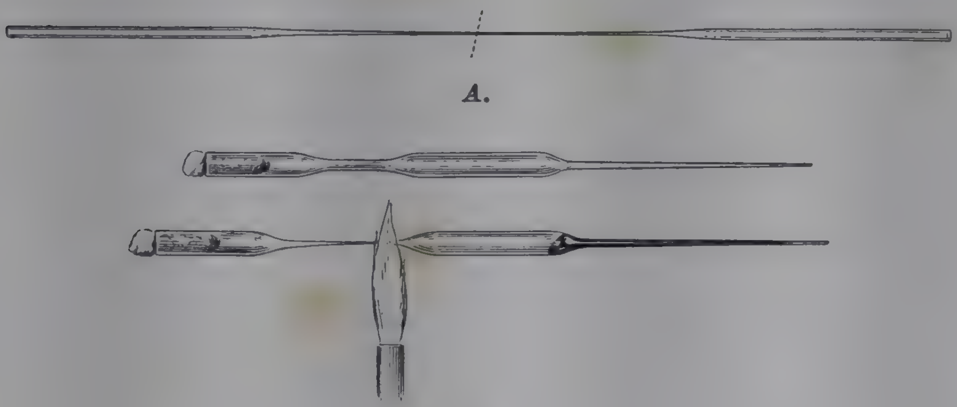

Fig. 328.—*A*, Cutting the capillary. *B*, Sealing the capillaries.

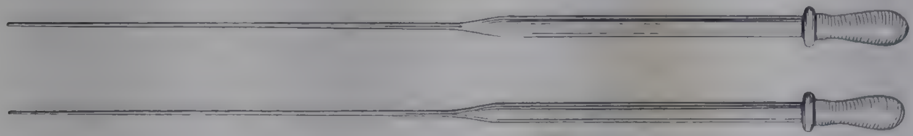

Fig. 329.—Completed capillaries.

The stylet should be kept in the needles. To insure protection of the point of the needle, it is well before sterilization to thrust a small piece of cork into the end of the needle.

Needles are governed by the law of deterioration as other material is. Repeated heating and use tend to weaken them. The newer the needle, the less danger of breakage. Test the temper of the large needle by bending it back and forth; short needles are best tested by pressing against the thumb.

The routine method of sterilization of needles is by dry heat for 2 hours at 170° C. The needles are placed in small test tubes, tightly stoppered, or they may be wrapped in paper which is thoroughly sealed. Objections to boiling are that sometimes infection has occurred from contamination of needles by spore-bearing organisms which are not killed by boiling.

Preparing Sterile Capillary Tubes

It is always well to have on hand in the laboratory pieces of glass tubing about 18 inches long, of 7 mm. caliber. These are prepared as follows: Cut a number of these tubes into 18 inch lengths. Stopper them at each end with a piece of cotton. Wrap them in separate bundles in paper and sterilize in the dry heat oven at 170° C. for two hours.

When a sterile capillary tube is required, open the package, remove one tube, heat it over a Bunsen burner (Fig. 327), and draw it out to the desired length and thickness. By turning the tube in the flame, a uniform degree of heat is obtained. When at least three inches are well heated, under rotation, the tube is taken out of the flame and rapidly pulled in a straight manner. It is then cut. These tubes can be made then into capillaries for drawing up blood or they can be used as sterile droppers.

Sterilizing in the Autoclave

One must learn to operate his own autoclave from operating directions and experience. Certain general rules, however, are of value. We shall describe the method of sterilizing in one type of steam pressure sterilizer:

The pop valves (safety valves at the top of the steam chamber) must be open at the beginning of sterilization. Fill the water tank below the sterilizer with water until the gauge is about two-thirds full. Be sure the water is turned off so that there is not a slow leak of water into the tank. Never fill the gauge completely. If the gauge is filled by mistake, the water can be drained from below the tank. Close all the valves on the sterilizer; that is, the chamber valve, the vacuum valve, and the valve on the door of the autoclave. Light the gas. Allow the pop valves to remain open until a good volume of steam comes from both of them. The steam should come in a steady stream, not in spurts. If it comes in spurts, there is air mixed with it. Now close the pop valves.

Allow the steam pressure to rise to 18 to 20 pounds in the jacket. If it goes above this, the safety valves will pop. When the pressure is 18 to 20 pounds in the jacket, open both the chamber and the vacuum valves simultaneously, using both hands. Allow a vacuum of 10 inches to register on the gauge above the autoclave, then immediately close the vacuum valve. Allow the pressure to rise in the steam chamber to 20 pounds. Both the chamber and jacket gauges should register the same pressure. Turn the gas low so as to maintain just 20 pounds pressure, and allow sterilization to proceed for thirty minutes. High pressure alone does not insure sterilization. The temperature must reach a proper degree. Good autoclaves are, therefore, equipped with thermometers. At 15 pounds pressure, e.g., the temperature should be 120° C.

Turn off the gas and allow the pressure to drop of its own accord to *O*. Never open any valves until there is no pressure in the autoclave.

Now open the door of the autoclave, and also open the pop valves. Always open the door while the sterilizer is still hot, but be careful not to be burned by the steam. Always leave all valves closed when through sterilizing.

After sterilization, drain the water out of the tank and refill two-thirds full of fresh water.

Place **tubes** in wire baskets to autoclave. When contaminated materials (used media) are sterilized, place the tubes and plates in deep trays or in buckets, so that the melted agar does not clog the pipelines.

Used tubes may be autoclaved in buckets, into which they are put upside down after removal of the plugs. The plugs should be destroyed by burning.

Protect the plugs of **bottles** by caps of wrapping paper which are fastened around the neck of the bottles with string. Cork and rubber plugs should not be used for glassware sterilized in the autoclave. Tubes which are to be closed with rubber stoppers must be sterilized with cotton in the autoclave. Sterilize the rubber stoppers separately in large test tubes, and exchange them for the cotton plugs under sterile precautions after autoclaving.

After emptying the autoclave, leave the sterile, unused tubes to solidify in the desired position. Immediately pour out the used media into proper containers (waste pails).

Petri dishes are best sterilized in buckets. The plates are opened, and the halves put into the bucket separately, upside down, so that the contents are liquefied by the heat and will flow to the bottom of the bucket. After autoclaving, the buckets are allowed to cool. The contents will solidify and can be easily removed.

Disposable plastic Petri dishes are available. These are sterilized by the manufacturer and do not have to be autoclaved if used only once.

Different **media** require differing pressures for various lengths of time. It is essential not to oversterilize, because the media suffer by prolonged or too intensive heating. The length of time necessary for sterilization also varies with the amount of media in the flasks. It is essential that baskets not be packed too tightly with tubes.

It is important, if **media containing fermentable substances** (commonly referred to as "sugars" or "carbohydrates") are to be sterilized before use, that the autoclave not be exhausted, but that the steam pressure be allowed to drop slowly. The best method is to shut off the steam immediately after sterilization and allow the autoclave to cool. The media are taken out when the temperature has dropped to 30° C. or lower, and the steam pressure in the chamber is equal to that outside.

Flowing steam is produced by leaving the door of the autoclave open. Careful watching of the thermometer is necessary to be certain that the inside of the autoclave reaches the proper temperature. The temperature may vary in different parts of the inner chamber, depending upon the location of the steam inlets.

Inspissation is the coagulation of media containing proteins (mostly serum or eggs). In order to obtain a smooth surface of the tubed media, great care must be exerted. Some laboratories use special inspissators, others perform this task in the autoclave. In the latter case, slant the tubes on a tray or rack and cover with newspaper. Then close the exhaust and the air outlet valve. Lock the door and let steam in slowly, to 15 pounds pressure. After 10 minutes slightly open the air outlet so that the pressure remains constant between 14.75 to 15.25 pounds. Sterilize for 20 minutes more, then shut off the steam and let the autoclave cool before opening the door.

Alli* suggested the following method: Fill a baking pan with fine sand to about 1 inch height. Cover the sand with a cloth and slant the tubes on the cloth. Place another cloth over the tubes and cover the first row with sand. Place more rows in the same manner. Open the steam valve and the air outlet until air is expelled. Slowly tighten the air valve until the pressure reaches 10 to 12 pounds, then close it. Maintain 15 to 17 pounds pressure for 30 minutes.

Sterilizing in the Dry Heat Oven

Never pack any sterilizer too tightly with material. Be sure the door is tightly closed before sterilization. Light the gas, keep the air outlet at the top of the oven open until the temperature registers 100° C., then close the outlet. Permit the temperature to rise to 170° C., and keep it at this point for two hours. After turning off the gas, be sure not to open the door until the contents have cooled to almost room temperature. Always time the sterilizer after the temperature reaches 170° C.

Pipettes and Petri dishes are best sterilized in appropriate metal boxes.

Recent investigations have been made concerning temperature levels in hot air ovens. These indications by Darmady and Brock[1] were made to assess, first, whether the heat actually penetrates to syringes to be sterilized in these hot air ovens; second, whether type of packing employed was satisfactory; and, third, at what position in the oven should the thermocouple of an automatic recording apparatus be placed. Usually to test an oven with thermocouples they are placed in glass tubes while the oven is empty. The efficiency of the oven is gauged by the degree of correlation of the temperature levels recorded throughout the oven. In England it is the custom to use a temperature of 160° C. for one hour as advocated by the Medical Research Council* on the sterilization of syringes. While Gradwohl advises 170° C. for two hours in a gas oven as being satisfactory, more recently Gerhards, using a fan oven, has shown that spores can be satisfactorily destroyed at 140° C. for one hour.

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*Mil. Surg. 95: 235, 1944.

[1] Darmady, E. M., and Brock, R. B.: J. Clin. Path. 7: 290, 1954.

In the present investigation, two gas ovens which were used showed wide variations of temperature. Generally, the temperatures were considerably lower in the bottom shelf than in the top. Syringes tested near the back wall were consistently overheated. Tightly packed syringes did not reach the top degree and as much as 60° C. differences were noted. At the end of one hour, syringes on the lower shelf, except those placed in the back, had not reached 160° C. Even after one hour there was a variation of 40° C.

With the exception of fan ovens, the majority of ovens do not meet all the criteria necessary for perfect sterilization. Of course tight packing is to be avoided, and there must be a free flow of air all around articles to be sterilized; metal boxes or similar sealed containers may prevent the heat's penetrating to those syringes near the center. Overloading ovens seriously interferes with the heat distribution.

These investigators found that with an electric oven without a fan the over-all variation of temperature was not as great as with a gas oven. These two investigators claim that gas ovens showed such wide variations of temperature that these instruments were unsatisfactory for syringe service.

Experiments with four electric ovens without fans showed the main heat is taken up slowly and that, even though close correlations of temperature are obtained when the oven is empty, wide variations of temperature may occur. Loading ovens while still hot does not interfere with the transfer of heat to the articles to be treated. In fact, in the majority of instances it is to be preferred, but tight packing of syringes and needles is condemned as unreliable.

Method of Using the Arnold Steam Sterilizer

Place the material to be heated in the Arnold steam sterilizer, and close both doors.

There should be a thermometer in the top of the sterilizer.

Fill the basin under the sterilizer with water.

Light the gas burner under the sterilizer, and maintain a constant temperature of 100° C. by keeping the burner wide open.

The steam will rise from the boiling water in the basin into the sterilizer.

Do not begin timing the sterilizer until the temperature reaches 100° C.

After sterilization, allow the temperature to drop to the temperature of the room before opening the doors of the sterilizer.

Sterilizing by Filtration, and Care of the Filters

The filter candle, placed in its mantle, is inserted through the hole of the rubber stopper which is to be used in the filtering flask. Care is taken that the candle is screwed tightly in the stopper, and that the rubber plug which is part of the apparatus fits tightly, so that there is no air leak.

Place the filter candle and mantle assembled in the rubber stopper into the filtering flask, taking care that the rubber stopper fits the flask. Plug the arm of the flask with nonabsorbent cotton, and attach a piece of rubber tubing. Sterilize in the autoclave at 15 pounds pressure for thirty minutes.

When cool, pour the material to be filtered into the mantle.

Attach to the rubber tubing of the filter a piece of glass tubing having a stopcock in the center of the tube.

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*Memorandum No. 15, 1945.

Attach to the suction pump.

Open the glass stopcock.

Start the suction, slowly at first, increasing gradually.

After filtration begins, alternately open and close the stopcock until the pressure is regulated in such way as to prevent foaming of the filtrate. When the pressure seems to be great enough for filtration to continue at a moderate rate, close the stopcock, and allow filtration to continue until the rate becomes considerably slower, then open the stopcock. After filtration, sterilize the Berkefeld filter as follows:

Autoclave at 15 pounds pressure for 30 minutes, then boil the filtering candle and mantle in a beaker or casserole before the fluid in the candle has been allowed to dry.

After boiling, remove with a pair of forceps, and dry in the incubator at 37° C.

After the candle has been dried, it is ready to be assembled, sterilized, and used for further filtration.

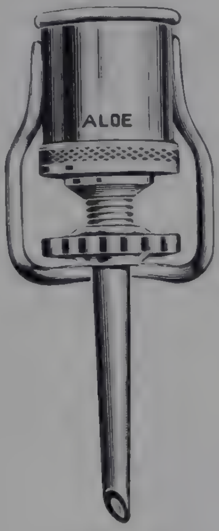

Fig. 330.

Fig. 331.

Fig. 330.—Seitz filter. (Courtesy A. S. Aloe Co., St. Louis, Mo.)
Fig. 331.—Berkefeld filter candle. (Courtesy A. S. Aloe Co., St. Louis, Mo.)

Filtration Through Seitz Filters

The Seitz filter makes use of asbestos pads instead of diatomaceous earth or unglazed porcelain for removal of bacteria from liquids during the filtration process. The asbestos filters are disk-shaped and vary in size and in grades of porosity. For bacterial work specify the E. K. grade of porosity when ordering the disks.

To use the Seitz filter assemble the apparatus by fitting the asbestos disk into the special metal container. Below the asbestos disk there should be placed a metal screen which comes with the apparatus. The screen and the disks are placed in the metal container and then tightened by means of set screws. The metal container with the asbestos in place is now covered with tinfoil then fitted tightly into the opening of a one hole rubber stopper which is placed in a Pyrex filtering flask. The arm of the filtering flask is plugged with nonabsorbent cotton and the whole apparatus is sterilized in the autoclave at 15 pounds pressure for 30 minutes. The apparatus as assembled is now attached to a vacuum pump in the same manner as the Berkefeld filter.

When filtration begins if there is foaming around the asbestos disk simply tighten the screws which hold it in the metal shield.

After use the disks are either burned or autoclaved and then discarded.

To Dispose of Contaminated Material

The best way to dispose of stools, sputa, etc., which are contained in paper or cardboard receptacles, and of contaminated wooden spatulas, swabs, etc., is to use an incinerator.

Place all other contaminated material in the autoclave preferably in deep trays or pails, to avoid overflow.

Sterilize at 15 to 20 pounds pressure for thirty to forty-five minutes (see pages 1394, 1395).

After sterilization has been completed, wash all glassware thoroughly with soap and water. If the glassware remains dirty, boil in a solution of Oakite,* one teaspoonful to a quart of water, and do not permit the water to dry on the glassware. Durlacque cleaner, which may be obtained at most grocery stores, is excellent for cleaning glassware. Any good detergent may be used.

Rinse thoroughly in tap water until all traces of the cleaning agent have been removed. It is very important to remove all the soap solution before sterilizing, or the glassware will become coated over with a white precipitate which is impossible to remove.

Immerse in a 1:1,000 nitric acid solution for several hours.

Rinse thoroughly in tap water to remove all traces of the acid.

Rinse in distilled water.

Wrap in the usual manner.

Sterilize in the dry heat oven at 170° C. for two hours.

Alternate Method.—Sterilize material as above. Pour out the hot contents into a waste pail. Discard plugs into incinerator pail. Place dishes and tubes in Charlab solution for 18 to 24 hours. Rinse with tap water, then proceed as above.

Care of the Incubator

To Regulate the Temperature at 37° C.

Attach incubator to the electric light socket, and turn the switch on. Allow the incubator to heat for twenty-four hours.

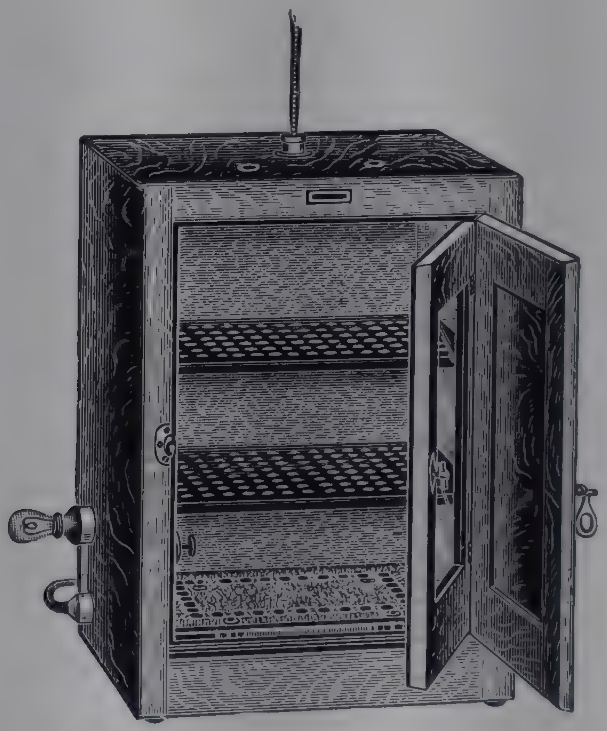

Fig. 332.—Electrically controlled incubator. (Courtesy E. H. Sargent & Co.)

If the temperature rises higher than 37° C., turn the thermostat wheel in the direction shown by the arrow next to the word "cold" until the light connected to the thermostat goes out.

Allow the door to remain closed until the light automatically turns on again, then read the thermometer. If the temperature at which the light turns on is higher than 37° C., turn the wheel again in the same direction, a slight turn, and proceed in this manner until the incubator maintains a temperature of 37° C.

If the temperature drops below 37° C., turn the wheel in the opposite direction.

Be careful to turn the thermostat wheel very slightly.

While the thermostat light is on, the incubator is heating.

When the light is off, the incubator is cooling. By watching this light, and the corresponding temperature changes, the incubator may be regulated fairly easily.

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*Oakite Products, Inc., 826 Clark Ave., St. Louis Mo.

Keep the incubator in the laboratory in a place where it will not be agitated.

Be sure to keep a container of fresh, clean water in the incubator at all times to prevent the air from becoming dry. The size of the water container and the amount of water needed depend on the size of the incubator.

STAINING PROCEDURES[1]

General Principles

All bacterial dyes are aniline or coal-tar dyes, synthetic products. They are called either "acid" or "basic" dyes. This does not mean that the dye itself is necessarily either a free acid or a free alkali, but that they are salts of these free color acids or bases. Acid dyes owe their colored properties to the anion. basic dyes, to the cation. The actual reaction of a dye in solution depends on the relative proportions of the dye ion and cation or anion with which it is combined in the dye salt.

Basic dyes have a greater affinity for nuclei, while acid dyes have an affinity for cytoplasm. Since nuclear material of bacterial cells seems to be distributed throughout their bodies, basic dyes are usually used to stain them.

Preparation of Smears

Smears from pure cultures of bacteria are usually prepared by making an aqueous suspension of the organisms, drying a drop of this on a slide or cover glass, and fixing by gentle heat. Because of their extremely tiny size and their stiff walls, bacteria do not undergo the shrinkage and distortion such a method of fixation would cause were higher forms of life used. A small amount of the material is removed and suspended in a drop of distilled water on a slide, taking care not to make too thick a preparation. It should be of a faint turbidity, and solid masses of bacteria should be avoided. A drop of a broth culture is sometimes used to make smears, but solids from the culture material may interfere with the stain, and for this reason this method is not always satisfactory. If a smear shows no areas where the bacteria are separated from each other, more water should be added to dilute the smear.

Several smears may be made on the same slide. A slide with frosted edges is used. It is divided into the necessary number of fields by lines drawn with a wax pencil. A corresponding number of lines is drawn with a graphite pencil on the frosted part of the slide. The code numbers of the smears made on the slide are written on the frosted surface with the same pencil. Eight to twelve smears may be stained on the same slide.

Several methods of fixing are in use. Preferred method is to dry the smear, then pass it rapidly through a Bunsen flame two or three times; or to allow the smear to dry on a flat, moderately hot plate of nonrusting metal on a boiling water bath. Overheating (burning) may be prevented by careful checking of the temperature of the slide, by just touching the back of the slide to the back of the hand each time the slide has been passed through the flame. Staining without fixation after air-drying is not recommended. Special staining methods may call for special fixing methods, in which case the method will be given.

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[1] Condensed from "Pure Culture Study of Bacteria" published by the Commission on Standardization of Biological Stains, May, 1944.

New slides must be used for acid-fast and flagella stains. It is essential that, after staining, the back surfaces of the slides be wiped clean with a piece of cloth or a paper towel.

Staining Formulas

Most of the staining technics given below are intended for use in study of pure cultures; various common staining formulas used in routine laboratory diagnosis of disease are also given. The literature contains many inaccuracies in staining methods. Few can be accepted in the light of present knowledge without some degree of interpretation to make them more explicit. In some cases the author's intentions are evident, while in others they must be inferred. Blind adherence to a staining technic is no guarantee for satisfactory results. *Always check a technic on known organisms for controls,* checking the solutions and water to make sure they are free from bacteria and spores.

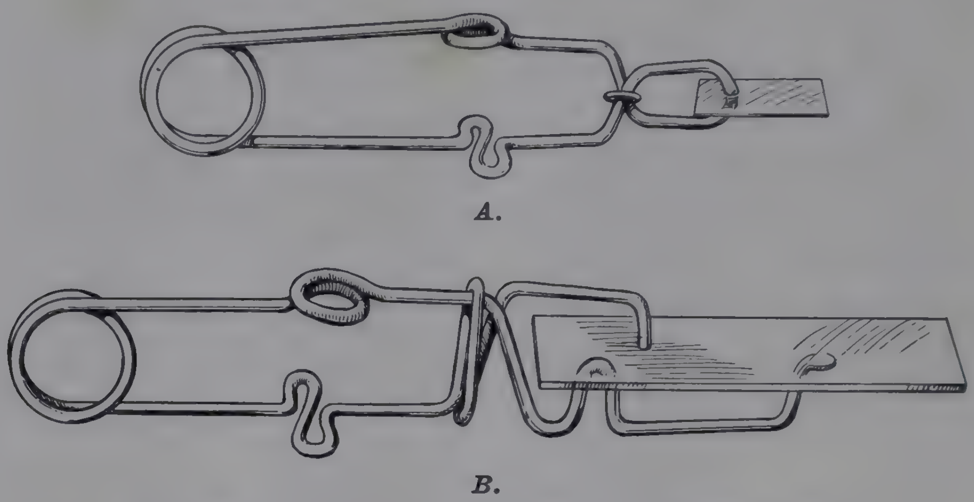

Fig. 333.—*A,* Cover glass holder. *B,* Slide holder.

STAINING TECHNIC*

Preparation and Fixation of Bacteriologic Smears

Slides should be fat-free, and free from lint or any other foreign substance.

Place a loopful of distilled water in the center of the slide.

Flame the platinum wire.

Remove the plug from the culture tube, or lift the cover of the Petri dish, or other container, flame the mouth of the container, hold the stopper between the third and fourth fingers.

With the cooled loop (which has been cooled inside of the tube or other container) pick up a portion of the material to be smeared.

Replace the stopper, flaming it, and flaming the mouth of the container.

Holding the slide in one hand, gently emulsify the material on the platinum loop in the water or saline on the slide.

Flame the loop.

The material on the slide should dry immediately.

Fix the smear by passing it three times through the flame, holding it in the upper portion of the flame a second at a time.

Allow to cool before staining.

If fixation by alcohol instead of dry heat is desired, the slide is placed in a Koplin jar of methyl alcohol for five minutes, or in ethyl alcohol for twenty minutes. Other fixatives, such as bichloride of mercury and acetic acid, may be used, the time varying according to the fixative.

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*Some staining methods not included in this section may be found throughout the text. Consult the index for other stains.

Used slides are placed in Lysol or some other disinfectant solution. Boil them in tap water for one to two hours, then clean in the same manner as slides for hematologic work. See page 612. When possible, do not use a slide more than once.

Stains, Staining Solutions, and Staining Technic

Points to Be Considered in Making Staining Solutions

1. Stains used should preferably be those bearing the certification label of the Biological Stain Commission. This Commission does not manufacture nor sell stains, but tests and certifies those sent to it voluntarily by various manufacturers. This Commission was formed through the efforts of many investigators; its executive committee consists of representatives of a number of scientific societies.

2. All dyes should be weighed accurately on an analytical balance.
3. The dyes should be ground in a mortar with the diluent, adding a small amount of the diluent at a time until all has been added.
4. All staining solutions should be filtered before use, but not before standing for at least twenty-four hours.
5. No solutions should be used after precipitation has occurred. When precipitation occurs, discard the solutions from the bottom of the bottle after the rest of the stain has been used, or filter and use the filtrate.
6. Diluted solutions should be made in small quantities. The stock solutions keep for a much longer period of time than the diluted solutions, particularly those containing either carbolic acid or potassium hydroxide.
7. All solutions should show on their labels the concentration of the dye present and the date of preparation. Use stain-fast labels.
8. Keep all staining solutions out of direct sunlight.
9. Keep all staining solutions in glass-stoppered bottles.

BACTERIOLOGIC STAINS

Formula numbers are given at the left of each formula; namely, S-1, S-2, etc.

Stock Solutions for Making Bacteriologic Stains*

S-1. Saturated Alcoholic Solution of Gentian Violet.—

Gentian violet	5.0 gm.
95% alcohol	100 c.c.

Grind the gentian violet in a mortar, adding the alcohol slowly, until all the dye is in solution.

Place in a glass-stoppered bottle, and filter through filter paper after 24 hours.

S-2. Saturated Alcoholic Solution of Basic Fuchsin.—

Basic fuchsin	3.5 gm.
95% alcohol	100 c.c.

Carry out the technic above.

S-3. Saturated Alcoholic Solution of Methylene Blue.—

Methylene blue	1.5 gm.
95% alcohol	100 c.c.

Carry out the technic above.

S-4. Lugol's Stock Solution.—

This solution is 15 times the strength of Gram's iodine solution, and may be used as a stock from which Gram's iodine is made.

Iodine, resublimed	5.0 gm.
Potassium iodide, c. p.	10.0 gm.
Distilled water	100 c.c.

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*This is not intended to be a complete catalogue of staining solutions. Other staining solutions are found throughout the text. Consult the index for special stains.

Rub up the iodine and the potassium iodide in a mortar until they are thoroughly mixed. Add the water, a small amount at a time, and grind it into the mixture, until all the water has been added. Allow to stand in a glass-stoppered bottle, and filter after twenty-four hours.

S-5. 10% Solution of Potassium Hydroxide.—

Dissolve 10 grams potassium hydroxide, c.p., sticks in about 50 c.c. distilled water in a stoppered Pyrex flask.

Cool under running water if the flask becomes hot.

Dilute to 100 c.c. with distilled water.

S-6. 5% Solution of Carbolic Acid.—

This may be made from the carbolic acid crystals or from liquid carbolic acid.

Liquid carbolic acid is made by adding 4 c.c. distilled water to each ounce of melted carbolic acid crystals.

If the crystals are used, they are melted, and 5 c.c. of the melted carbolic acid are used in 100 c.c. solution.

If the liquid carbolic acid is used, 5.74 c.c. of the liquid carbolic acid are used in 100 c.c. solution.

Place 50 c.c. distilled water in a 100 c.c. graduated cylinder.

Add either 5 c.c. *liquefied* carbolic acid crystals or 5.74 c.c. *liquid* carbolic acid.

Dilute to 100 c.c. with distilled water.

S-7. 1% Solution of Carbolic Acid.—

This may be made from the 5% solution by diluting one part of 5% carbolic acid solution to a total of 5 parts, using distilled water.

If made from the melted crystals, 1 c.c. of liquefied carbolic acid crystals is added to 50 c.c. distilled water in a 100 c.c. graduated cylinder, and then diluted to 100 c.c. with distilled water.

Diluted Stains

These dilutions are the stains actually used in staining bacteria. They are made from the above stock solutions.

Carbol Gentian Violet

S-8. Made From Stock Saturated Alcoholic Solution.—

Add 10 c.c. saturated alcoholic solution of gentian violet to 50 c.c. 1% carbolic acid solution in a 100 c.c. graduated cylinder.

Dilute to 100 c.c. with 1% carbolic acid solution.

Filter after twenty-four hours.

S-9. Made From Gentian Violet Crystals.—

Gentian violet	1 gm.
Carbolic acid crystals	2 gm.
Absolute alcohol	10 c.c.
Distilled water to make	100 c.c.

Rub up the gentian violet and the alcohol in a mortar.

Add the carbolic acid crystals, and mix.

Add two-thirds of the water, a small amount at a time, stirring constantly.

Pour the mixture into a bottle, and rinse the mortar with the remaining water, adding it to the mixture in the bottle.

Filter through filter paper after twenty-four hours.

S-10. Hucker and Conn.—

20% solution of crystal violet in 95% alcohol	100 c.c.
1% solution of ammonium oxalate, large crystals, in distilled water	100 c.c.

Mix just before use equal parts of the two solutions.

S-11. Kopeloff and Bermann.—

1% gentian violet *or* crystal violet *or* methyl violet 6B in distilled water	150 c.c.
5% solution of sodium bicarbonate in distilled water	50 c.c.

Just before use mix 15 c.c. of the violet solution with 4 c.c. of the bicarbonate solution.

S-12. Scudder.[*]—

1% solution of crystal violet in distilled water	90 c.c.
Phosphate buffer, pH 6.9 (see page 1407, S-49)	10 c.c.

Carbol Fuchsin

S-13. Made From Stock Saturated Alcoholic Solution.—

Add 10 c.c. saturated alcoholic solution of basic fuchsin to 50 c.c. 5% carbolic acid solution in a 100 c.c. graduated cylinder.

Dilute to 100 c.c. with 5% carbolic acid solution.

Filter after twenty-four hours.

S-14. Made From Basic Fuchsin Crystals.—

Basic fuchsin	1 gm.
Carbolic acid crystals	5 gm.
Absolute alcohol	10 c.c.
Distilled water to make	100 c.c.

Proceed as above as for carbol gentian violet.

S-15. Randolph and Mickell.[1]—

1% solution of basic fuchsin in propylene glycol	100 c.c.
5% solution of carbolic acid in distilled water	400 c.c.

Just before use, mix 10 c.c. of the fuchsin solution with 40 c.c. of the carbolic acid solution. Shake well.

S-16. Diluted Carbol Fuchsin.—

Dilute carbol fuchsin 1:10 with distilled water. Use the same day.

Methylene Blue

S-17. Loeffler's, Made From Stock Saturated Alcoholic Solution.—

Distilled water	100 c.c.
10% solution of potassium hydroxide	0.1 c.c.
Saturated alcoholic solution of methylene blue	30 c.c.

Filter through filter paper after 24 hours. When a "certified stain" is used, the addition of potassium hydroxide is optional.

S-18. Loeffler's, Made From Methylene Blue Crystals.—

Distilled water	100 c.c.
10% solution of potassium hydroxide	0.1 c.c.
Methylene blue, certified	0.3 gm.
95% alcohol	30.0 c.c.

Rub up the methylene blue with alcohol in a mortar. Transfer to a flask. Add the potassium hydroxide to the water and wash the mortar with the mixture. Pour the washings into the flask.

Filter through filter paper after 24 hours.

S-19. Manson's Methylene Blue.—

Methylene blue	2 gm.
Borax (sodium tetraborate crystals)	5 gm.
Distilled water	100 c.c.

Dissolve the borax in the distilled water.

Grind the methylene blue in the borax solution, a small amount at a time, and pour into a bottle.

Filter after one week.

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[*] Stain Technol. **19**: 39, 1944.

[1] Am. Rev. Tuberc. **49**: 39, 1944.

S-20. Gabbett.—

Methylene blue	2 gm.
25% sulphuric acid	100 c.c.

Place the methylene blue in a bottle. Pour the acid over it. Filter after 24 hours.

Safranin

S-21. 1% Aqueous Solution of Safranin.—

Dissolve 1 gram of safranin in 100 c.c. distilled water by grinding it in a mortar, and adding the water, a small amount at a time, until all the dye has been dissolved.

Filter after 24 hours.

S-22. 0.25% Solution of Safranin.—

Safranin	0.25 gm.
95% alcohol	10 c.c.
Distilled water	90 c.c.

Rub up the safranin with the alcohol in a mortar. Pour into a flask. Wash the mortar repeatedly with small amounts of the distilled water. Pour washings into the flask. Filter through filter paper after 24 hours.

Neutral Red-Fuchsin, Meyrick and Harrison[1]

S-23. 1% solution of neutral red in distilled water	150 c.c.
Carbol-fuchsin	10 c.c.

Bismarck Brown, Neisser

S-24. Dissolve 0.2 gm. Bismarck brown Y in 100 c.c. of boiling distilled water. Filter through filter paper while still hot.

Gram's Iodine

S-25. Made From Stock Lugol's Solution.—

Add 10 c.c. Lugol's solution to about 100 c.c. distilled water in a graduated cylinder.

Dilute to 150 c.c. with distilled water, and mix.

Filter after 24 hours.

S-26. Made From Iodine Crystals.—

Iodine, resublimed	1 gm.
Potassium iodide, c.p.	2 gm.
Distilled water	300 c.c.

Proceed as for Lugol's solution, S-4, above.

S-27. Kopeloff and Bermann.—

Weigh 4 gm. sodium hydroxide into a 500 c.c. glass-stoppered flask.

Add 50 c.c. distilled water.

Shake until dissolved.

Add 10 gm. iodine, resublimed.

Shake until dissolved.

Dilute to 500 c.c. with distilled water.

Let stand in a dark place for 24 hours, then filter through filter paper into a brown glass-stoppered bottle.

Carbol Thionin, Nicolle

S-28. Thionin	1.0 gm.
Phenol	2.5 gm.
Distilled water	100.0 c.c.

Filter; dilute before use with an equal quantity of distilled water.

Pyronin Green

S-29. Pappenheim.—

Methyl green, certified	1.0 gm.
Pyronin Y, certified	0.25 gm.

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[1] J. Path. & Bact. 54: 517, 1942.

Phenol	2.0 gm.
95% alcohol	5.0 c.c.
Glycerin	20.0 c.c.
Distilled water	100.0 c.c.

Mix the alcohol and phenol.
Dissolve the methyl green in alcohol-phenol.
Mix glycerin and distilled water.
Dissolve the pyronin in it.
Mix the two solutions.

S-30. Scudder.*—

Methyl green, ethylated	0.65 gm.
Pyronin, Y, certified	0.1 gm.
Distilled water	100.0 c.c.

Dissolve dyes in order.
Filter after 24 to 48 hours.

Nigrosin

S-31. Nigrosin Solution, Dorner.—

Nigrosin†	10 gm.
Distilled water	100 c.c.

Boil 30 minutes in an Erlenmeyer flask; then add as preservative:

Formalin (40%)	0.5 c.c.

Filter twice through double filter paper and store in serologic test tubes, about 5 c.c. to the tube. Stopper tubes with tinfoiled corks.

Neisser Stain

S-32. Solution I.—

Methylene blue	0.1 gm.
Absolute alcohol	2.0 c.c.
Glacial acetic acid	5.0 c.c.
Distilled water	95.0 c.c.

Dissolve the methylene blue in the absolute alcohol in a mortar.
Mix the acetic acid with the distilled water.
Add the acetic acid mixture to the solution of methylene blue a small amount at a time.
Filter after 24 hours.

S-33. Solution II.—

Crystal violet, certified	1.0 gm.
95% alcohol	10.0 c.c.
Distilled water	300.0 c.c.

Just before use mix 20 c.c. of Solution I and 10 c.c. of Solution II.

S-34. Solution III.—

Dissolve 2 gm. chrysoidin in 300 c.c. hot distilled water.
Filter while still hot.

Ljubinsky Stain

S-35.	Crystal violet, certified	0.25 gm.
	Glacial acetic acid	5.0 c.c.
	Distilled water	95.0 c.c.

Albert Stain

S-36.	Toluidine blue	0.15 gm.
	Glacial acetic acid	1.0 c.c.
	95% alcohol	2.0 c.c.
	Distilled water	100.0 c.c.

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*Stain Technol. 19: 39, 1944.

†Nigrosin B Grubler recommended by Dorner; American nigrosins certified by Biological Stain Commission usually satisfactory.

Dissolve the toluidine blue in the alcohol.
Mix the acetic acid with the water.
Add the acetic acid solution to the dissolved dye.
After 24 hours filter through filter paper.

Wayson Stain

S-37.

Basic fuchsin	0.2	gm.
Methylene blue	0.75	gm.
Absolute alcohol	20.0	c.c.
Phenol crystals	10.0	gm.
Distilled water	200.0	c.c.

Dissolve the fuchsin and the methylene blue in the alcohol.
Dissolve the phenol in the distilled water.
Mix the two solutions.
After 48 hours filter through filter paper.

Richards and Miller Stain

S-38. Stain.—

Phenol, liquefied	3.0	c.c.
Distilled water	87.0	c.c.
Auramine 0	0.1	gm.
95% alcohol	10.0	c.c.

Mix the phenol and the water.
Dissolve the dye in the alcohol.
Add the phenol water to the dye solution.
Filter, if necessary.

S-39. Decolorizer.—

Sodium chloride	0.5	gm.
Concentrated hydrochloric acid c.p.	0.5	c.c.
70% alcohol	100.0	c.c.

Dissolve the salt in the acid.
Add the alcohol.

Pappenheim Differentiating Stain

S-40.

Corallin, yellow (rosolic acid)	1.0	gm.
Methylene blue	about 7.0	gm.
Absolute alcohol	100.0	c.c.
Glycerin	20.0	c.c.

Dissolve the corallin in the alcohol.
Filter after 48 hours.
Add the methylene blue.
Let stand for 4 days, shaking frequently.
Filter through filter paper.
Add the glycerin.
After 24 hours filter through filter paper.

Cooper's Solutions

S-41. Cooper Carbol Fuchsin.—

To 100 c.c. carbol fuchsin prepared according to formula S-13 or S-14, add 3 c.c. of a 10% solution of sodium chloride in distilled water. Keep in the refrigerator.

S-42. Alkaline Brilliant Green.—

To 100 c.c. of a 1% solution of brilliant green in distilled water add 1 c.c. of a 1% solution of sodium hydroxide in distilled water.

Marshall Stain[1]

S-43. Picric Acid Solution.—

Mix 10 c.c. of acetone with 50 c.c. of absolute alcohol.

Add picric acid to saturation. Filter through filter paper after 48 hours.

S-44. Auramine Green.—

Auramine O	0.09	gm.
Distilled water	90.0	c.c.
Malachite green	1.1	gm.
Distilled water	110.0	c.c.

Dissolve the auramine in 90 c.c. distilled water and mix.

Dissolve the malachite green in 110 c.c. distilled water and mix.

Mix the two solutions.

After 48 hours filter through filter paper.

Stains for Rickettsiae and Inclusion Bodies

Ordway-Machiavello[2]

S-45. Regaud's Fixative.—

3% potassium bichromate in distilled water	80 c.c.
Formalin	20 c.c.

Prepare fresh for use.

S-46. Stain.—

1% Poirer's blue, color index No. 707, in distilled water	10 c.c.	Mix.
0.45% eosin, bluish, color index No. 771, in distilled water	15 c.c.	Mix.

Mix the two solutions just before use.

Add 25 c.c. of distilled water slowly, with constant shaking.

Use within 24 hours. The dyes and the water used in this stain must be neutral.

Nyka[3]

S-47.

Basic fuchsin	5.0	gm.
90% alcohol	100	c.c.

Before use dilute 1.0 c.c. with 9.0 c.c. of distilled water.

S-48.

Methyl violet 7B	0.1	gm.
Distilled water	100	c.c.

Castañeda

S-49.

A. Buffer Solution.—

Dissolve 23.86 gm. disodium phosphate ($Na_2HPO_4.12H_2O$) in 1 liter of distilled water.

Dissolve 11.34 gm. anhydrous monosodium phosphate (NaH_2PO_4) in 1 liter of distilled water.

To 88 c.c. of the disodium phosphate solution add 12 c.c. of the monosodium phosphate solution, mix, and filter through a Berkefeld or Seitz filter.

Add 0.2 c.c. Formalin.

B. Methylene Blue Solution.—

Dissolve 21 gm. methylene blue in 300 c.c. of 95% alcohol.

Dissolve 0.1 gm. potassium hydroxide in 1 liter of distilled water.

Mix each solution, then after 24 hours add solution A to solution B and mix.

For further stains for Rickettsiae, see pages 1413, 1414; for Negri bodies, see page 1720.

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[1] Brit. M. J. 2: 232, 1943.

[2] Lab. Digest 6: 2, 1942.

[3] J. Path. & Bact. 56: 264, 1944.

Capsule Stains

Hiss

S-50.	Saturated alcoholic solution of gentian violet	5 c.c.
	Distilled water	95 c.c.

MacNeal

S-51.	Eosin Y, certified	1.0 gm.
	Methylene blue, certified	1.0 gm.
	Azure A	0.6 gm.
	Methylene violet, Bernthsen	0.2 gm.
	Methyl alcohol, neutral, acetone-free	1,000.0 c.c.

Heat to 50° C for 30 minutes.

Keep at 37° C. for two days.

Filter through filter paper.

It is preferable to purchase the stain in dry form or in solution.

Gray*

S-52. Mordant.—

Saturated watery solution of mercuric chloride	10 c.c.
20% solution of tannic acid in distilled water	10 c.c.
Saturated watery solution of potassium alum	25 c.c.

S-53. Stain.—

Methyl alcohol	10 c.c.
1% solution of basic fuchsin in distilled water	10 c.c.

Mix.

Methyl alcohol	5 c.c.
1% solution of methylene blue in distilled water	5 c.c.

Mix.

Add fuchsin solution to methylene blue solution.

Lawson**

S-54. Mordant.—

5% solution of phosphomolybdic acid in distilled water.

S-55. Stain.—

Dilute 10 c.c. of Wright stain with 5 c.c. of glycerin just before use.

Flagella Stains

Gray

S-56.	Saturated solution of potassium alum in distilled water	5.0 c.c.
	20% solution of tannic acid in distilled water	2.0 c.c.
	Saturated aqueous solution of mercuric chloride	2.0 c.c.
	Saturated alcoholic solution of basic fuchsin	0.4 c.c.

Mix in order just before use.

Leifson

S-57.	5% solution of potassium alum in distilled water	10 c.c.
	2% solution of tannic acid in distilled water	10 c.c.
	1% solution of basic fuchsin in 95% alcohol	10 c.c.

Mix in order.

We have found it desirable to purchase the stain in powdered form from the Baltimore Biological Laboratory.

Dissolve 1.5 gm. of BBL Flagella stain in a mixture of 35 c.c. of 95% alcohol and 65 c.c. of distilled water.

Allow to stand for 10 minutes shaking several times.

The stain remains stable for several weeks if kept in a tightly stoppered bottle.

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*J. Bact. 45: 301, 1943.

**J. Lab. & Clin. Med. 25: 435, 1940.

Decolorizing Agents

Distilled Water.—Distilled water is a decolorizing agent. If water is allowed to remain on slides after they have been stained, it will remove part of the stain. After staining and washing preparations, always stand them on end to dry if they cannot be blotted.

Absolute Alcohol.—Absolute alcohol is sometimes used as a decolorizing agent. For the Gram stain 95% alcohol is recommended.

Acid Alcohol.—Add 3 c.c. of concentrated hydrochloric acid, c.p., to enough 95% alcohol to make a total volume of 100 c.c.

Various acids and alkalies act as decolorizing agents. Where a special decolorizing solution must be used, it will be described with that staining technic.

STAINING METHODS

Simple Stains for Morphology

These stains serve to study the general morphology of the microorganisms. The slides are fixed over the flame before staining.

(I) Methylene Blue.—

Use methylene blue prepared according to directions under "methylene blue" (S-17) (S-18). For staining microorganisms in blood films, Manson's methylene blue (S-19) is used.

Flood the slide with methylene blue and stain for 1½ to 3 minutes.
Wash with distilled water.
Dry by draining, and examine.

(II) Dilute Carbol Fuchsin.—

Flood slides with diluted carbol fuchsin (formula S-16) for ½ to 1 minute.
Wash with distilled water.

(III) Carbol Thionin (formula S-28).—

Use as methylene blue.

Negative Demonstration of Bacteria

India ink, diluted with equal parts of distilled water or nigrosin (formula S-31) is used.

Mix a loopful of the bacterial suspension on the slide with the same amount of India ink or nigrosin solution. Make a very thin smear. Examine after drying.

Gram Stain

The Gram stain permits the differentiation of two groups of organisms, one of them called "gram-positive," the other "gram-negative." With most Gram staining methods, the gram-positive organisms stain purple, while gram-negative organisms stain red. While the Gram stain is the most commonly used bacterial stain, there is no uniformity in its technic. Because of the danger of overstaining or overdecolorizing, it is recommended that at least once a day known gram-negative and gram-positive organisms be stained at the same time as the cultures which are being examined. The following methods of Gram staining technic are commonly used:

(I) Original Gram Stain.—

Fix the slide in the flame and allow to cool.
Stain with carbol-gentian-violet (formula S-8 or S-9) ---------------------60 seconds
Wash with distilled water.
Stain with Gram iodine (formula S-25 or S-26) --------------------------30 seconds
Wash with distilled water.

Decolorize in 95% alcohol until no further violet comes away.

Wash thoroughly with distilled water.

Counterstain with 1% aqueous safranin (S-21) --------------------------2 minutes

Wash with distilled water.

Dry in the air, and examine.

Gram-positive organisms stain purple.

Gram-negative organisms stain red.

(II) Hucker Modification.—

Fix the slide in the flame and allow to cool.

Stain with Hucker crystal violet (formula S-10) ----------------------60 seconds

Wash with distilled water.

Stain with Gram iodine (formula S-25 or S-26)-------------------------60 seconds

Wash with distilled water.

Decolorize with 95% alcohol until no further violet comes away.

Wash thoroughly with distilled water.

Counterstain with 0.25% safranin (formula S-22)-----------------------10 seconds

Wash with distilled water.

Dry in air and examine.

(III) Kopeloff and Bermann Modification.—

This is an excellent stain for vaginal and urethral smears.

Fix the slide in the flame and allow to cool.

Stain with Kopeloff violet (formula S-11)-----------------------------5 minutes

Pour off stain.

Flood slide two or three times with Kopeloff iodine solution (formula S-27).

Decolorize with 100% acetone until no further violet comes away.

Dry in the air.

Counterstain with freshly prepared 1:1,000 watery basic fuchsin *or* neutral red-fuchsin Meyrick and Harrison (formula S-23)-----------------------------20 to 30 seconds

Wash with distilled water.

Dry in the air and examine.

No water should be used in this procedure before the application of fuchsin.

(IV) Scudder Modification.—

Fix the slide in the flame and allow to cool.

Stain with Scudder crystal violet (formula S-12)---------------------3 to 5 minutes

Pour off stain.

Apply Kopeloff iodine (formula S-27) two or three times.

Decolorize with 100% acetone until no further violet comes away.

Dry in air.

Counterstain with Scudder methyl green-pyronin (formula S-30)------1 to 2 minutes

Wash with distilled water.

Dry in the air and examine.

Scudder's modification of the Gram stain is used to study the cytologic elements present in pus.

(V) The Counterstain of Pappenheim.—

Stain as when carrying out any good Gram procedure. Preferred method is that of Kopeloff and Bermann (III).

Instead of fuchsin, counterstain with Pappenheim pyronin-methyl green (formula S-29) --1 minute

This method permits study of the cytology of smears from secretions and excretions.

Wash with distilled water.

Dry in air and examine.

(VI) Gram Stain for the Color-Blind.—

Stain with any accepted modification.

Instead of safranin, pyronin, or fuchsin, counterstain with Bismarck brown (formula S-24) --30 to 60 seconds

Gram-positive organisms are purple.

Gram-negative organisms are yellowish-brown.

Staining Acid-Fast Organisms

Acid-fast organisms are usually slow in taking stains but they do not lose them when treated with diluted acids and alcohol. When phenol is used as a mordant, heating is necessary to accomplish the staining. Instead of heating, however, cold staining for 24 hours may be substituted. Carbol fuchsin is the usual stain for the acid-fast organisms. With most staining methods the acid-fast organisms stain red.

(I) Ziehl-Neelsen Stain.—

Fix the slide in the flame.

Cover with carbol fuchsin (formula S-13 or S-14) and heat the slide gently from below with a Bunsen burner so that steam is visible (but not to boiling) ------------3 minutes

If the carbol fuchsin evaporates, add fresh solution.

Wash with distilled water.

Decolorize with 95% alcohol containing 3% hydrochloric acid until no further red comes away.

Wash with distilled water.

Either counterstain with methylene blue (formula S-17 or S-18)------1½ to 2 minutes *or* with 2% watery solution of malachite green *or* with brilliant green S-42--------2 minutes

Wash with distilled water.

Dry in the air and examine.

Acid-fast organisms are red.

Cells and other bacteria are blue (when methylene blue is used as a counterstain) or green (when malachite green is used as a counterstain).

(II) Cooper Modification.—

Fix the slide in the flame.

Stain with Cooper carbol fuchsin (formula S-41)----------------------3 to 5 minutes gently heating the slide so that steam develops.

Wash with distilled water.

Decolorize with 5% nitric acid in 95% alcohol.

Wash with distilled water.

Counterstain with Cooper brilliant green (formula S-42)----------30 seconds or longer

Wash with distilled water.

Dry and examine.

(III) Modification for Color-Blind Workers.—

Fix the slide as above.

Cover with carbol gentian violet (formula S-8 or S-9) and heat the slide to steam—5 minutes.

Wash with distilled water.

Decolorize with 95% alcohol containing 5% nitric acid until no further red comes away.

Counterstain with Bismarck brown (formula S-24)------------------------2 minutes

Wash with distilled water.

Dry in the air.

Acid-fast organisms are purple.

Cells and other bacteria are yellowish-brown.

(IV) Gabbett Modification.—

Fix the slide, stain with carbol fuchsin and wash with distilled water as above.

Decolorize and counterstain at the same time with Gabbett methylene blue solution (formula S-20) --20 to 30 seconds

Wash with distilled water.

Dry in the air and examine.

(V) Fluorescent Dye Method.—(See pages 1609 ff.)

Place a yellow filter in the ocular. The microscope lamp is equipped with a blue filter which transmits ultraviolet light. Use an aluminized mirror. Carry out the examination in a darkened room, using the 8 mm. objective and 20x oculars.

Stain the slide with Richards and Miller stain (formula S-38)--------2 to 3 minutes
Wash with tap water.
Decolorize with decolorizing solution (formula S-39) for 10 minutes or longer.
Wash with tap water.
No counterstain is used.
Dry in air and examine.

(VI) Pappenheim Method.—

Fix slide and stain with carbol-fuchsin as under Method I.
Do not wash.
Pour on Pappenheim differentiating stain (formula S-40) four or five times.
Wash with distilled water.

Dry in the air and examine. This method stains *Mycobacterium tuberculosis* but not the smegma bacillus. Tubercle bacilli are red, while smegma bacilli usually appear blue.

(VII) Randolph and Mickell Cold Method.—

Fix slide in flame.
Cover with Randolph and Mickell carbol fuchsin-propylene glycol (formula S-15)------------------4 minutes or longer
Wash with water.
Decolorize, counterstain and carry out the remainder of the procedure as under Method I.

(VIII) Simultaneous Gram and Acid-Fast Stain of Marshall.—

Fix slide over flame.
Stain with carbol fuchsin (formula S-13 or S-14)-------------------------5 minutes gently heating so that steam develops.
Wash with distilled water.
Apply picric acid solution (formula S-43) just to decolorize.
Wash with distilled water.
Stain with 0.25% crystal violet solution in distilled water---------------------1 minute
Wash with distilled water.
Cover with Lugol iodine (formula S-25 or S-26)---------------------------1 minute
Decolorize with 100% acetone.
Wash with distilled water.
Counterstain with auramine green (formula S-44)------------------15 to 30 seconds
Wash with distilled water.
Blot, examine.

Myco. tuberculosis stains crimson red with black granules; *Myco. smegmatis*, grayish green. Gram-positive organisms are purple-black; gram-negative organisms, grass-green.

Granule Stains

These stains serve for the study of bacterial morphology. They accentuate the granules in the bodies of *Corynebacterium diphtheriae* and are, therefore, most frequently used for studies of throat and nose smears.

Smears from the lesions or from cultures must be dried and fixed over the flame before the granule stain is applied.

(I) Methylene Blue.—

Apply methylene blue (formula S-17, S-18, or S-19)----------------1 to 2 minutes.
Wash with distilled water.
Dry in air and examine.
Granules are deep blue, bodies lighter.

(II) Gohar.*—

Stain with methylene blue (formula S-17)-------------------------------5 minutes.
Quickly decolorize with 1:1,000 sulphuric acid.
Rapidly wash with distilled water.

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*J. Bact. 47: 575, 1944.

Stain with Lugol iodine (formula S-25 or S-26)----------10 seconds.
Wash with distilled water.
Counterstain with 1% eosin in distilled water----------30 seconds.
Wash with distilled water.
Dry in air and examine.
Granules are dark; bodies, yellowish.

(III) Neisser Technic No. I.—

Stain with Neisser solution I (formula S-32)----------30 to 60 seconds.
Wash with distilled water.
Stain with Bismarck brown (formula S-24)----------10 seconds.
Wash with distilled water.
Dry in air and examine.

(IV) Neisser Technic No. II.—

Stain with Neisser mixture (formula S-32 and S-33)----------10 seconds.
Wash with distilled water.
Counterstain with Neisser solution III (formula S-34)----------10 seconds.
Wash with distilled water.
Dry and examine.
Granules are dark; bodies, yellow.

(V) Ljubinsky.—

Stain with Ljubinsky stain (formula S-35)----------2 minutes.
Wash with distilled water.
Stain with Bismarck brown (formula S-24)----------30 seconds.
Wash with distilled water.
Dry and examine.
Granules are deep purple; bodies, brownish.

(VI) Albert.—

Stain with Albert stain (formula S-36)----------5 minutes.
Drain, do not wash.
Apply Lugol iodine solution (formula S-25 or S-26)----------30 seconds.
Wash briefly with distilled water.
Dry and examine.

Staining Rickettsiae and Inclusion Bodies

In many cases, staining with Giemsa or Wright solution carried out as with blood films, or the hematoxylin-eosin stain will yield good results. Special stains are used for Negri bodies (see page 1720). Out of the many dozens of suggested modifications, the following yielded good results in the hands of the editor:

(I) Ordway-Machiavello.—

Fix tissue in Regaud's fixative (formula S-45) 4 days, changing the fluid every day.
Wash in running water for 16 to 24 hours.
Transfer to 70% alcohol.
Carry through the routine histologic technic employing the paraffin method.
Cut sections not over 5 microns thick.
Deparaffinize.
Wash well in several changes of water.
Stain with Ordway-Machiavello stain (formula S-46)----------6 to 8 minutes.
Decolorize with 95% alcohol until pale bluish-pink.
Wash in absolute alcohol----------1 minute.
Transfer to two changes of xylol, slightly washing in the first.
Mount from second xylol.
Rickettsiae and inclusion bodies are bright red; nuclei and cytoplasm, sky blue.

(II) Nyka.—

Fix in Mueller fluid.
Carry through routine paraffin method.
Deparaffinize.
Keep in distilled water for 5 minutes.
Stain with Nyka first stain (formula S-47)------------------------5 to 10 minutes.
Rinse with tap water.
Differentiate with 90% alcohol ------------------------------------1 to 2 minutes.
Stain with Nyka second stain (formula S-48)----------------------1 to 3 minutes.
Rinse and differentiate with 1:1,500 acetic acid.
Dehydrate and mount.

(III) Castañeda.—

This is best for Rickettsiae.
Stain with Castañeda stain (formula S-49)-----------------------2 to 3 minutes.
Wash with running water---30 seconds.
Counterstain with safranin (formula S-21)----------------------1 to 2 minutes.
Drain and examine.

Capsule Stains

The capsules of bacteria are often detectable as unstained halos surrounding the bodies of the organisms. In many cases, however, special staining methods are necessary to ascertain the presence or absence of the capsules. The methods listed below give good results.

(I) Hiss.—

Mix material to be stained with an equal amount of serum. Make thin smear.
Allow to dry in air.
Fix either in the flame or in 1:10 diluted commercial Formalin.
Cover with Hiss stain (formula S-50) and heat to steaming for a few seconds.
Wash off the stain with a 20% watery solution of copper sulphate.
Dry in air and examine.
Bacterial bodies, cells, and background stain purple.
Capsules are colorless or pale lavender.

(II) MacNeal.—

Make thin smear.
Allow to dry in air.
Cover with MacNeal tetrachrome stain (formula S-51)----------------3 to 15 minutes.
Wash carefully with distilled water.
Dry in air and examine.
Capsules are colorless or pale.

(III) Lawson.[1]—

Prepare direct smear, without using a diluent, from the culture.
Dry in air.
Cover film with mordant (formula S-54)---------------------------------30 seconds.
Wash the slide with methyl alcohol.
Cover with 20 drops of stain (formula S-55)---------------------------2 minutes.
Add 30 drops of distilled water, mix stain on slide with the water and allow to act----
--------10 to 20 minutes.
Rinse with distilled water.
Drain slide and examine when dry.
Capsules are colorless or pale.

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[1] J. Lab. & Clin. Med. 25: 435 1940.

Staining Flagella

General Method of Applying Flagella Stains

Very young and vigorous cultures must be procured. In general, twelve to twenty-four-hour growths on surface of agar are satisfactory. If stock cultures are used, make daily transplants for several days before studying, to restore vigor. Kulp* in 1926 suggested this method:

Use agar slants with plenty of water of condensation. Transfer a loopful of this water of condensation from a twenty-four hour culture to a second slant, then twenty-four hours later pour off one or two drops of condensation water aseptically into a tube of sterile distilled water of the same temperature as the culture. Incubate at optimum temperature of the organism for forty-eight to seventy-two hours. Johnston and Mack recommended a similar method. Others find that bacteria have a tendency to shed their flagella if left in water that long, one member of the Commission[2] obtaining excellent results when bacteria were in the water only seven hours.

Cover glass or slide must be scrupulously clean. Place in hot cleaning solution (page 192), clean in strong sodium or potassium hydroxide, rinse in weak hydrochloric acid, then in distilled water. Keep in alcohol until ready for use. Burn off alcohol from cover glasses just before use, holding cover glasses in the flame with forceps. Plimmer and Paine recommend baking slides on a wire gauze mat over a Bunsen burner just before using.

Greatly dilute the growth in distilled water, place tiny drops or streaks on the cover slip; discard those showing signs of grease. Preparations should be thin enough to dry immediately. Fixation of films by heat, although recommended by some authors, may destroy the flagella. Plimmer and Paine recommend scrupulously clean slides instead of cover slips, heated before use, and cooled to body temperature. Using a 3 mm. loop, place a drop of culture fluid on one end of the slide and tilt to allow the suspension to run down the slide. Slide should be warm enough to allow rapid drying. Gray placed a large loopful of the culture suspension on one end of a slide, and spread gently with a strip of unsized paper (e.g., typing paper).

Poor results are caused by greasy cover glasses or slides, old cultures, unsatisfactory medium, too slow drying of the smears, too thick suspensions of bacteria, insufficient or excessive mordanting, understaining, or overstaining. Most poor results come from improper handling of the culture, or from greasy slides or cover slips. The personal equation also enters into the picture. Best results seem to have been obtained with the Leifson and Gray methods, although success has been reported where other methods were used.

(I) Gray.—

Prepare film as directed in General Method and dry.
Apply Gray mordant (formula S-56)--------------------------------5 to 10 minutes.
Wash with distilled water.
Stain with carbol fuchsin (formula S-13 or S-14)-------------------5 to 10 minutes.
Wash with distilled water.
Drain and examine when dry.

(II) Leifson.[3]—

Prepare three slides.

With a wax pencil make a heavy continuous line along the edges of the slides, leaving about ¾ inch on one end free for handling. Place a large loopful of bacterial suspension on one end and tilt the slides so that the suspension runs down.

Dry in the air.

Add to each slide 0.8 to 1.0 c.c. of Leifson stain formula (S-57).
Stain first slide --8 minutes.
Second slide ---10 minutes.
Third slide --12 to 15 minutes.

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*Stain Technology 1: 60, 1926.

[2] Biological Stain Commission.

[3] J. Bact. 36: 656, 1938.

Wash with tap water.
Drain and examine when dry.
If counterstain is necessary, stain with 1:1,000 methylene blue in a 1:2,000 aqueous solution of borax------10 minutes.

Staining Spores

If a special method is not used but the Gram or a simple bacterial stain applied, spores appear as unstained holes in the bacterial body. In order to visualize spores more clearly, special methods must be applied.

(I) Moeller.—

Make thin smears and fix in the flame.
Cover with chloroform ------2 minutes.
Wash with distilled water.
Cover with 5% chromic acid ------½ to 2 minutes.
Wash in water.
Steam with carbol fuchsin (formula S-13 or S-14)------3 to 5 minutes.
Decolorize with 5% sulphuric acid ------5 to 10 seconds.
Wash in water.
Stain with methylene blue (formula S-17, S-18, or S-19)------½ to 1 minute.
Wash in water.
Dry and examine.
Spores are red; bodies of bacteria, blue.

(II) Abbott.—

Make thin smears and fix in flame.
Cover with methylene blue (formula S-17, S-18, or S-19) and heat, bringing the stain to boiling several times but not continuously------1 minute.
Wash in distilled water.
Cover with a mixture of 1 part of saturated alcoholic eosin solution and 9 parts distilled water ------5 to 10 seconds.
Rinse with distilled water.
Blot.
Mount in balsam or clarite and examine.
Spores are blue; bodies of bacteria, pink.

(III) Wirtz-Conklin.—

Make thin smears and fix in the flame.
Cover with 5% aqueous malachite green and steam------3 to 6 minutes.
Wash with distilled water.
Stain *either* with ½% aqueous safranin *or* 5% aqueous Mercurochrome--30 to 60 seconds.
Wash with distilled water.
Dry and examine.
Spores are green; bodies of bacteria, pink.

CULTURE MEDIA

Culture media are divided into collecting, preserving, plating, and diagnostic media. With the exception of a few very selective media, all media must be sterile. Heat sterilization, however, may harm certain ingredients. It is of paramount importance, therefore, to adhere rigorously to the directions concerning sterilization. The glassware in which the media are stored or used must be chemically clean and sterile. No traces of the cleaning solution must remain on the glassware. Dichromate-sulphuric acid mixtures are not recommended for this reason. The tubing of media and the pouring of plates must be carried out in a dust-free room. Speaking and walking in the laboratory are to be avoided during these operations.

Most media are stored in the refrigerator or in the icebox. Proper plugging protects against contamination and, partly, against evaporation. Dried-out media cannot be used because a certain water content is essential for the growth of most organisms. Smaller laboratories often dispense rarely used media into large test tubes and store them in the refrigerator until used. Then a plate is poured with the contents of one of these tubes. *It is necessary to substitute the water lost by evaporation during storage.* In order to know how much water was lost, mark the upper level of the medium on each tube with a wax pencil when the tubes are taken out of the sterilizer. If a tube is taken out of storage and the contents melted, sterile distilled water must be added to fill the contents of the tube to the pencil mark. Seventeen to twenty c.c. are the proper amounts of medium per 100-mm. plate, if not directed otherwise.

Each tube or plate must be correctly labeled with a wax pencil, immediately after sterilization, to indicate its content.

Media should not be reheated or resterilized more often than allowed in the formula.

In addition to proper composition and sterilization, the pH of the medium and the final reaction are very important. This is checked by the use of indicators.

Small laboratories should purchase media ready-made, tubed, and sterilized. Baltimore Biological Laboratory, Difco, Inc., and the Gradwohl Laboratories supply such media.

Every bacteriologist, clinical pathologist, and laboratory technician should realize that proper media are the most essential tools in bacteriologic work. Proper preparation and proper keeping of the media are of such importance that this cannot be emphasized too strongly.

General Facts Concerning Culture Media

Culture media are divided into fluid, semifluid, semisolid, and solid media according to their consistency. Isolated colonies can be obtained only on solid media. The other media serve either as collecting, preserving, and enrichment fluids, or for the study of pure cultures. Solid media, except for certain coagulated media, are frequently used in plates. They may be poured into Petri dishes so that the material to be examined can be spread on the surface. Or the material to be examined may be mixed with the melted medium and this mixture poured into plates and allowed to solidify. In this way colonies may be isolated and selected for further study.

Present-day technic uses agar as the chief solidifying agent. When 0.05 to 0.2 per cent of agar is added to the medium, the medium will be semifluid. Such a solution supports the growth of Neisseriae, Clostridia, etc. Semisolid tubes contain 0.3 to 0.5 per cent agar. While high concentrations of agar (2 to 3 per cent) are commonly used in Europe, most American methods require only 1.5 to 1.7 per cent agar for a solid plate.

Media containing agar become liquid when heated to about 96° C. and solidify again when the temperature falls to about 45° C. Tubes are placed in a boiling water bath to liquefy the medium. Sometimes it is necessary to melt the agar in a flask over a Bunsen flame protecting the flask with a wire-and-abestos net, as for example, for dissolving dehydrated media when preparing them for use. In such cases, it is necessary to shake the flask frequently to prevent the ingredients from sticking to the bottom of the flask. If the agar burns, the culture medium must be discarded.

Gelatin is rarely used today because it melts at around 20° C. and therefore ''cold incubation'' is necessary if it is to be kept solid. It is still, however, the most reliable measure of bacterial proteolysis.

Distilled water should be used for the preparation of culture media.

All chemicals must be chemically pure.

Most media contain peptone in one form or another. While Bacto-peptone and BBL-polypeptone satisfy most requirements, many special peptones are used. Pancreatic digest of casein (Difco) has been used recently as an all-purpose peptone. Difco-tryptone and BBL-trypticase are recommended for indol production; Difco-proteose-peptone and BBL-thiotone for hydrogen sulphide production; Difco-neopeptone, proteose, and tryptose have their special indications. BBL-phytone is a papaic digest of plant material. Most media require 0.5 to 2 per cent peptone. It is essential that the proper peptone be used for each medium. Peptones are easily dissolved in distilled water.

Meat is essential for many media. Meat extract (Difco, BBL, Wilson, or others) is used routinely, but for more fastidious bacteria, infusions of heart, liver, brain, etc., are indicated. Some authors suggest ''hormone'' and tryptic digest broths which have growth supporting properties. These hormone media require special care, and for that reason the ready-made commercial products are used in many laboratories. Meat extracts, however, often give false positive fermentation reactions and are, therefore, by-passed whenever possible in such media.

Yeast extract, Bacto-Casamino acids, and supplements are required for a number of media. Yeast extract (Bacto) 0.3 to 1 per cent, strongly supports the growth of many microorganisms and may be substituted for a part or all of the meat extract when so indicated in the formula.

Fermentable substances—carbohydrates, alcohols, glucosides, and urea—are often destroyed by heat. They are, therefore, frequently added to the previously sterilized ''base'' after filtration through a Berkefeld or Seitz filter.

High quality Bacto (Difco) and BBL products may be easily substituted for ''hormone'' and other digest media described in European literature, as well as European brands of peptones.

Hemolysis must never be evaluated on plates containing fermentable substances, notably dextrose or sodium azide, since these materials cause false readings.

Negative indol tests will sometimes be observed when media containing carbohydrates are used for this reaction.

Many laboratories use dehydrated (desiccated) media. Such media are very convenient, simple to prepare, and very reliable. Excellent desiccated media are available from Difco, Inc.,[1] and the Baltimore Biological Laboratory (BBL).[2]

The intention of this edition of *Clinical Laboratory Methods and Diagnosis* is to recommend relatively fewer media than the former editions and to concentrate on those which are easily prepared or readily purchased on the market.

A few points in the technic of media making should be clarified.

(A) Making Solutions.—

Peptones, agar, and gelatin often adhere to the walls or to the bottom of the flask, and burn when the flask is heated. This spoils the medium. Particles adhering to the neck of culture tubes may cause sticking of the cotton plug. To avoid the adhesion of these materials to the glass surfaces, observe the following precautions:

Pour a small amount of water in the flask before adding the ingredients. If, for example, the medium is to be made up by dissolving the ingredients in 1000 c.c. of water, first pour about 500 c.c. of the water into the flask, then add the ingredients. Finally, pour the rest of the water into the flask to wash the inside of the neck of the flask. This washes into the contents of the flask any powder adhering to the neck.

The capacity of the flask should be double the amount of the medium to be made. Erlenmeyer flasks with wide necks and without pour-outs are preferred.

(B) Sterilization.—

The autoclave must not be packed too tightly or sterilization will not take place. Cover the plugs of the flasks with wrapping paper tied with a string around the necks of the flasks. Sterilize test tubes standing in racks with the exception of media containing eggs or serum or other coagulable substances. These must be slanted during sterilization.

(C) Pouring Plates.—

Plates must be poured under sterile conditions. Repeatedly flame the necks of the flasks between pouring the plates. Hold the plug of the flask in such a position that it does not become contaminated.

The media should not be warmer than 60° C. when poured. This will reduce the formation of water of condensation.

With the exception of Brewer's anaerobic agar, it is best to leave the plates on the laboratory table until the media have solidified, then store the plates upside down in the refrigerator. Store Brewer's agar right side up.

Each plate must be marked to indicate its contents.

Never use dried-out plates or plates showing growth of contaminants.

If the surface of the plate is moist, put the plate, with partly removed lid, in a dust-free incubator until the surface is dry. Do not open the incubator during this procedure except to take out the drying plates.

Some very selective media, such as those used for stool work, may be dried on the laboratory table with partly removed lids.

(D) Tubing Media.—

(1) Media which are sterilized after they have been tubed.—

Attach a piece of rubber tubing to a short-stemmed 4 to 6 inch glass funnel. (Fig. 334.) The size of the funnel varies according to the amount of the medium. Insert an eye dropper into the other end of the rubber tubing so that the pointed end of the eye dropper is free. Place this tubing apparatus in a ring stand high enough that space remains to operate the outlet. Put a Moore or Cenco clamp on the rubber tubing to allow interruption of the flow, and close the clamp. Fill the funnel with the medium. To fill the tubes, place the eye dropper well into the tubes so that the medium will flow into the tubes without soiling the upper parts which come in contact with the plug.

Plug the tubes and sterilize them.

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[1] 720 Henry St., Detroit 1, Mich.

[2] 1640 Gorsuch Ave., Baltimore 18, Md.

(2) Media which are tubed under sterile conditions.—

The safest procedure is to use sterile pipettes and distribute the medium into sterile test tubes. It is best to have an assistant to flame the necks of the tubes and insert the plugs.

Another method is to prepare a tubing apparatus using a 500 c.c. (or larger) bottle, Pyrex glass, with a tube at the bottom. Adjust the rubber tubing and the eye dropper as they were adjusted to the funnel (1, above). Wrap the entire apparatus in paper and sterilize.

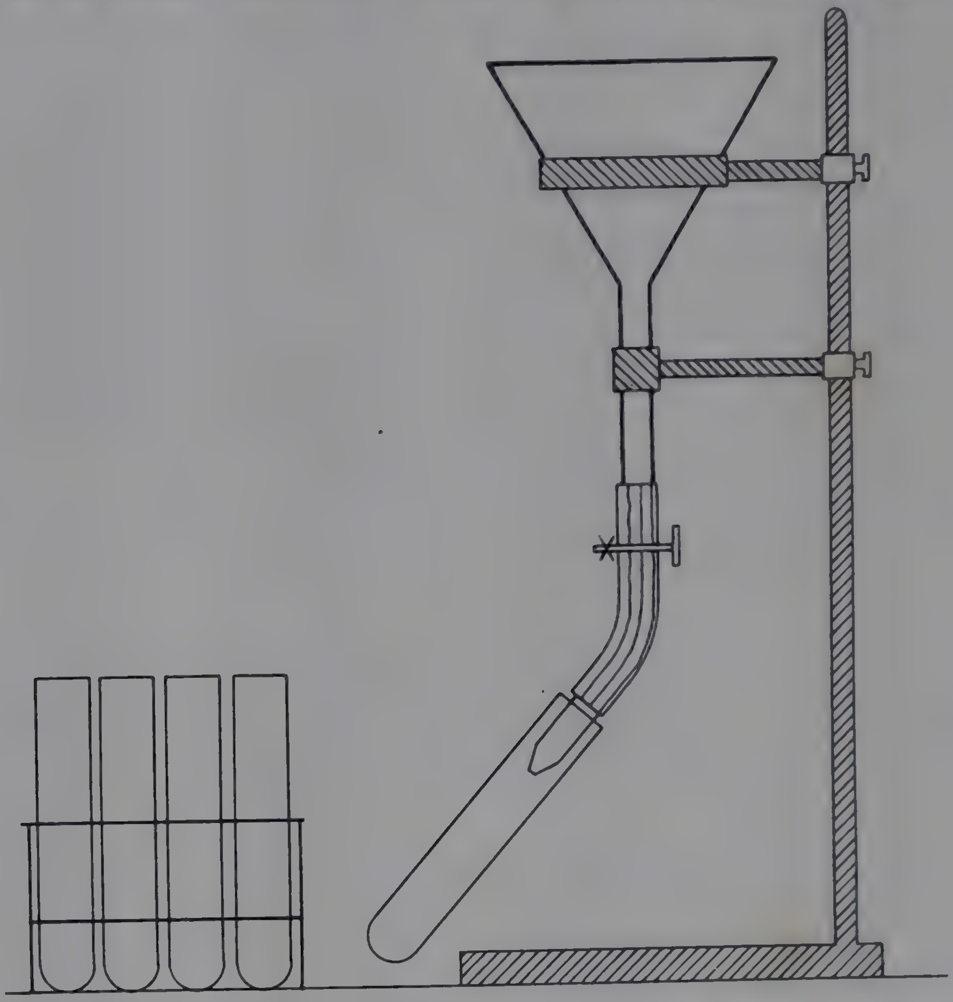

Fig. 334.—Apparatus for tubing culture media. (Courtesy Dr. Oscar Felsenfeld.)

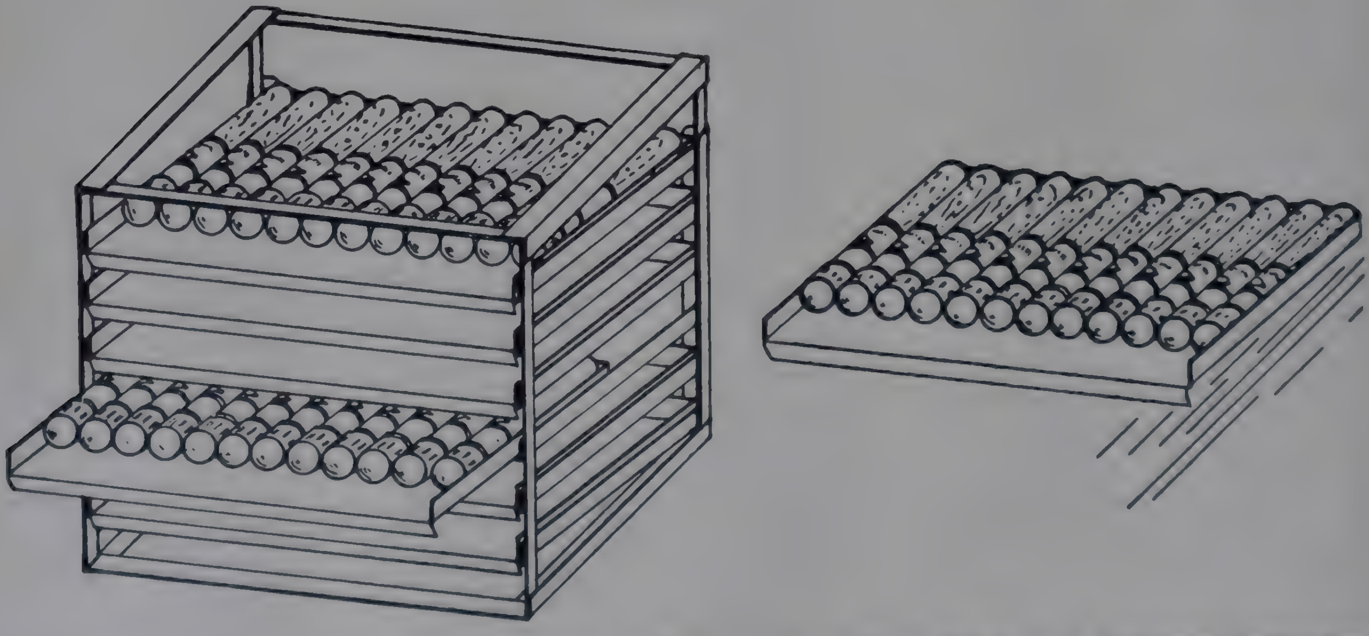

Fig. 335.—Bacteriologic tube cabinet, which retains culture tubes at a 5 degree angle for storage or incubation of slant surfaces in a horizontal plane. It may also be used for incubating fluid cultures when maximum surface contact is desired. (Courtesy Baltimore Biological Laboratory, Inc., Baltimore, Md.)

Protect the outlet of the apparatus against contamination during the operation by covering it with a sterile test tube between fillings.

All media tubed after sterilization must be checked for sterility by incubation at 37° C. for 24 to 72 hours, according to the medium.

(E) Preparation of Eggs.—

(1) Whole eggs.—

Scrub fresh eggs with soap, then wash with water, and put into 70% alcohol for 30 minutes or longer.

Prepare a large, strong-walled bottle containing numerous glass beads; plug with nonabsorbent cotton and sterilize in the dry heat oven.

Take the eggs out of the alcohol and wrap them in a sterile towel. When they are dry, open them with a sterile knife, nicking both ends, and collect the contents under sterile conditions in the sterile bottle. Shake the bottle until the eggs are homogeneous.

Take care not to touch the ends of the eggs after taking them out of the alcohol.

(2) Egg yolks.—

Clean and sterilize the egg shells as for the collection of whole eggs (1, above).

It is best for the operator to wear sterile rubber gloves. If he does not, then he must scrub his hands clean before handling the eggs.

Open the eggs by breaking the shell in the middle and parting the halves. Separate the yolks and the whites. Pour the yolks into the sterile bottle, and save the egg whites for other purposes.

Special Indicators Added to Culture Media

Andrade Indicator

One c.c. of Andrade indicator is used for each 100 c.c. of culture medium.

The medium containing the Andrade indicator is pink when hot, but becomes colorless when cooled to room temperature, if it is neutral or alkaline. It changes to a red or a deep pink when acid.

The method of preparation is given on the bottle if the acid fuchsin is purchased from a reliable chemical house. If the National Aniline Company's dye is used, dissolve 200 mg. of the acid fuchsin in 100 c.c. of distilled water.

It is suggested that if no technic of making the indicator is given on the label of the dye the worker use 500 mg. of acid fuchsin.

This solution turns a magenta red.

Add N/1 sodium hydroxide a drop at a time, and stirring after the addition of each drop until the solution turns from red, through pink, then to reddish brown, then to yellow.

The change in color is gradual, and the addition of the N/1 sodium hydroxide should be made very slowly, waiting several minutes after each few drops.

It usually requires 17 c.c. N/1 sodium hydroxide to bring the change in the color.

Phthalein Indicators

Use only reliable indicators; the Difco products are excellent.

(a) **Brom Thymol Blue.—**

Dissolve 0.4 gm. brom thymol blue
in 12.8 c.c. of N/20 sodium hydroxide by grinding in a mortar.
Dilute to 100 c.c. with distilled water.
Use 2 to 4 c.c. for each 1000 c.c. of culture medium.

(b) **Brom Cresol Purple.—**

Dissolve 0.4 gm. of brom cresol purple
in 13.8 c.c. of N/20 sodium hydroxide by grinding in a mortar.
Dilute to 100 c.c. with distilled water.
Use 2 to 4 c.c. for each 1000 c.c. of culture medium.

(c) **Phenol Red.—**

Dissolve 0.4 gm. phenol red
in 23.5 c.c. of N/20 sodium hydroxide by grinding in a mortar.
Dilute to 100 c.c. with distilled water.
Use 2 to 4 c.c. for each 1000 c.c. of culture medium.

Methods of Adjusting the Reaction of Culture Media*

Note.—N/10 solutions are always used in the titration of culture media to insure accuracy of results. N/1 solutions are used to adjust the reaction in order not to interfere too much with the total volume of medium.

(1) Colorimetric or Hydrogen Ion Method of Adjusting the Reaction of Culture Media to pH 7.2 to 7.6.—

Materials needed.—

Buret containing N/10 sodium hydroxide, filled to mark *0*.
0.02% phenol red indicator (aqueous). (Page 32.)
0.02% cresol red indicator (aqueous). (Page 32.)
1 c.c. pipette graduated in 0.1 c.c.
10 c.c. pipette.
Measuring cylinder, 100 c.c. capacity, graduated in 1 c.c.
Buffer solutions of pH 7.0, 7.2, 7.4, 7.6, 7.8, or an indicator color chart such as that found in Lubs: *The Determination of Hydrogen Ions* or Plate XL.
5 c.c. pipette for measuring the buffer solutions.
Test tubes for buffer solutions, and for the medium after it is adjusted.
Casserole.
N/1 sodium hydroxide solution.
N/10 hydrochloric acid.
Stirring rod.

Technic.—

Place 5 c.c. of each buffer in its respective tube, each tube labeled with the pH value of the buffer, and the name of the indicator. Add 0.5 c.c. 0.02% phenol red indicator to each tube.

Prepare a similar set of tubes, using cresol red indicator.

If the indicator chart is used, the buffer solutions may be omitted.

Place 10 c.c. of the medium to be adjusted in a casserole.

Add 1 c.c. phenol red indicator. If the medium is acid, it will turn yellow; if it is neutral, it will turn the orange color of the standard of pH 7.0 with phenol red indicator; if it is alkaline, it will turn red.

If it is acid, add the N/10 sodium hydroxide from the buret, a drop at a time, stirring constantly, until the solution in the casserole changes from yellow through orange to the red of the standard of pH 7.2, 7.4, 7.6 with phenol red indicator.

This is the end point. It may be desirable to place the medium in the casserole into a test tube to compare it with the buffer or the chart. Read from the buret the number of c.c. of N/10 sodium hydroxide used.

Calculation.—

Calculate the number of c.c. of N/10 sodium hydroxide required to adjust the reaction of the total quantity of culture medium made.

Divide this number by 10 to obtain the number of c.c. of N/1 sodium hydroxide to be used.

Add the N/1 sodium hydroxide to the medium, and mix thoroughly. Test the adjusted medium.

Example.—

It required 0.8 c.c. N/10 sodium hydroxide to adjust the reaction of 10 c.c. of culture medium. It will take 100 times 0.8 c.c., or 80 c.c., N/10 sodium hydroxide to adjust the reaction of 1000 c.c. culture medium. 1/10 of this quantity, or 8 c.c. of N/1 sodium hydroxide, is required to adjust the reaction of the medium. Add the 8 c.c. N/1 sodium hydroxide to the medium, and mix. The medium must be tested after the addition of the sodium hydroxide.

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*See hydrogen ion concentration, pp. 29 ff.

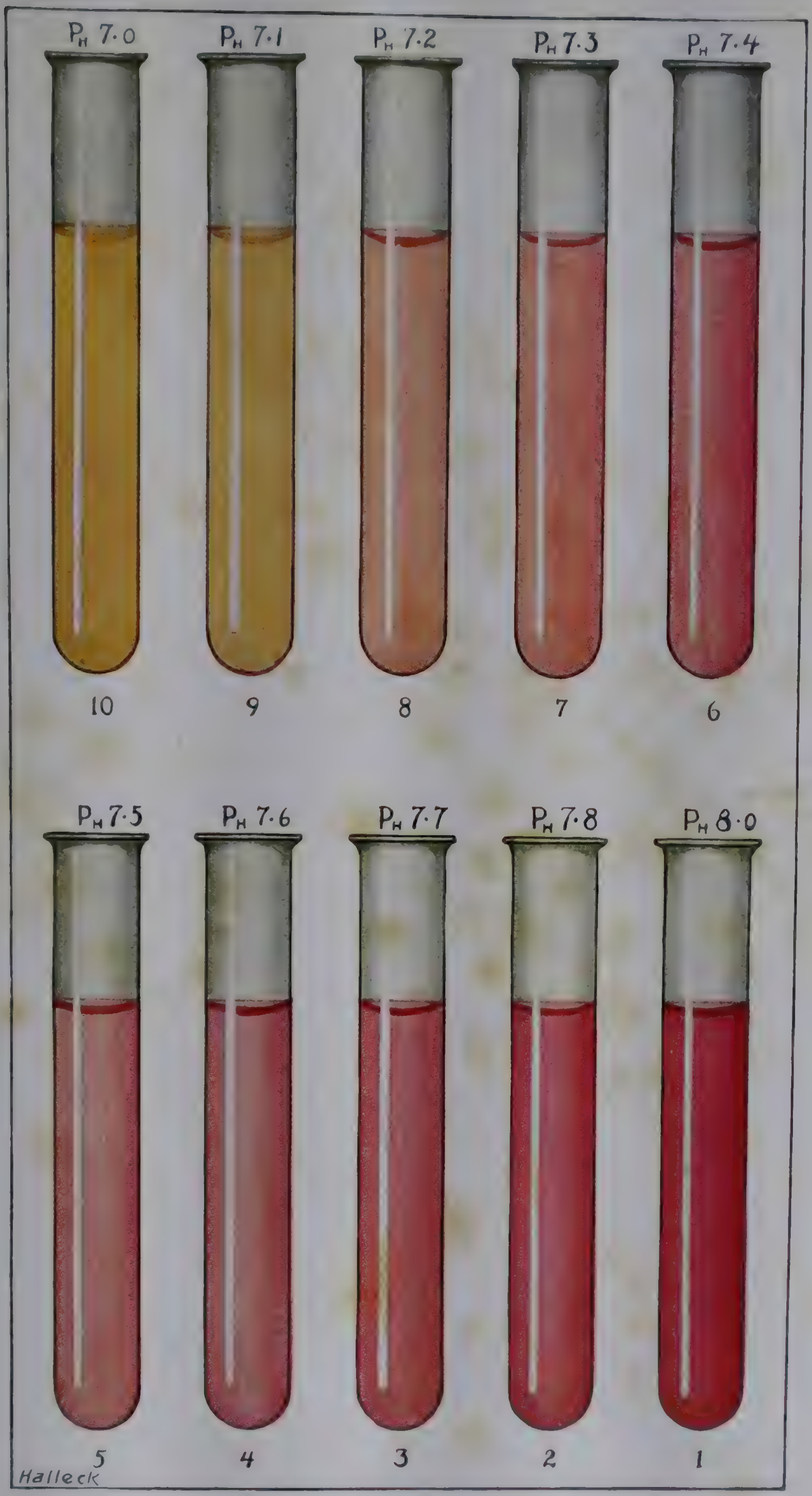

PLATE XL.—CHART SHOWING APPROXIMATELY THE TINTS PRODUCED BY ADDING PHENOLSULPHONEPHTHALEIN (PHENOL-RED) TO A SERIES OF PHOSPHATE SOLUTIONS OF THE H ION CONCENTRATIONS INDICATED IN EACH CASE BY pH.

(From Macleod: Physiology in Modern Medicine, The C. V. Mosby Co.)

Testing the Result.—

Place 5 c.c. of the adjusted medium in each of two test tubes.

To one of the tubes, add 0.5 c.c. of 0.02% phenol red indicator, and to the other, add 0.5 c.c. of 0.02% cresol red indicator.

Compare the colors of the two tubes with their respective standards. The tube containing the phenol red indicator should match the standard containing phenol red at the same point as the tube containing the cresol red indicator matches its standard. If this point is the desired end reaction, then the medium has been properly adjusted.

A solution at pH 7.6 reacts orange to phenol red and yellow to cresol red.

If too much sodium hydroxide has been added, titrate back to the desired pH, using hydrochloric acid in the buret.

(2) Colorimetric or Hydrogen Ion Method of Adjusting the Reaction of Culture Media to Other pH Values.—

The technic is the same as the above, but the end point is different. The sodium hydroxide is added to the medium in the casserole until it changes from yellow through orange to the red color of pH 8.0, as seen by matching with the color of the standard of that reaction.

After the medium has been adjusted, it reacts orange to cresol red, and red to phenol red.

If a medium of pH 7.0 is required, use phenol red as indicator because it changes color near this point. Alkalinize the fluid with N/10 sodium hydroxide until pink, then add N/10 hydrochloric acid until it turns just yellow.

(3) **Use of the potentiometer** is explained on pages 35 ff.

(4) **Use of indicator papers** is explained on pages 29 ff. and 43.

(5) **The pH of desiccated (dehydrated) commercially prepared media** is already adjusted. Any interference with it might spoil the medium.

Formulas of Culture Media

Formulas of culture media listed herein are numbered consecutively as M-1, M-2, etc., at the left of the medium.

M-1. Peptone Water.—

Peptone*	10 or 20 gm.
Sodium chloride	5 gm.
Distilled water	1000 c.c.

Dissolve the peptone and the salt in the water.

Filter through filter paper, if necessary.

Adjust the reaction to pH 7.4 to 7.6. For cholera work, pH 7.8 to 8.2 is favorable.

Distribute into test tubes or bottles.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

M-2. Meat Extract Broth.—

Peptone	10 gm.
Sodium chloride	5 gm.
Beef extract	3 gm.
Distilled water	1000 c.c.

Weigh the beef extract on a piece of wax paper.

Heat some of the water to 60° C. in a beaker. Drop the paper with the beef extract into the beaker.

Dissolve the salt and the peptone in some water.

Mix the beef extract solution and the salt-peptone solution.

Add the rest of the water. Filter through filter paper.

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*Instead of peptone, tryptose, tryptone, thiotone, polypeptone, trypticase, or neopeptone may be used.

Adjust the reaction to pH 7.2 to 7.4

Distribute into test tubes or flasks.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

BBL extract broth, 18 gm. desiccated powder dissolved in 1000 c.c. distilled water, may be used instead of the above formula.

M-3. Trypticase Soy Broth, BBL.—

Trypticase	17	gm.
Phytone	3	gm.
Sodium chloride	5	gm.
Dipotassium phosphate	2.5	gm.
Dextrose	2.5	gm.

Suspend 30 gm. of the powder in 1000 c.c. of distilled water.

Mix thoroughly.

Warm gently until solution is complete.

Dispense and sterilize in the autoclave at not over 15 pounds pressure (121° C.) for 15 minutes.

If desired, 0.5 to 1.0 gm. of agar may be added per liter of broth before sterilization.

M-4. Infusion Broth—From Meat, Heart, Liver, or Brain.—

(a) Chop meat, heart, liver, or brain, and mix 500 gm. of it for "single strength" or 1000 gm. for ''double strength'' infusion with 1000 c.c. distilled water, in an enamel or stainless steel pot.

Let stand at 4° to 6° C. overnight, in the refrigerator.

Skim off the fat with a metal or enamel spoon.

Heat to 45° C. in a water-bath and keep at this temperature for one hour.

Boil without stirring for 30 minutes.

Filter through glass wool.

Dilute to 1000 c.c. with distilled water.

(b) Add tryptose or trypticase	10 gm.;	double strength	20 gm.
sodium chloride	5 gm.;	double strength	10 gm.

Adjust the reaction to pH 7.4 to 7.6. Heat to the boiling point, allow to cool, and adjust the reaction, if necessary.

Distribute in test tubes or flasks.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

Bacto-Heart Infusion Broth With Tryptose or BBL-Infusion Broth With Thiotone is prepared by dissolving 25 gm. of the desiccated powder in 1000 c.c. of distilled water.

M-5. Broth With Fermentable Substances.—

(a) Add to each 100 c.c. of peptone water (formula M-1) or extract broth (formula M-2) or cystine trypticase base (formula M-6), after the reaction is adjusted, 0.4 c.c. of a suitable indicator, such as 0.4% brom thymol blue, brom cresol purple, or phenol red.

Distribute 5 c.c. amounts into test tubes, preferably with screw caps.

Sterilize in an autoclave for 20 minutes at 15 pounds pressure.

Ready-made broth bases for fermentation studies are available as Bacto or BBL phenol red broth base and purple broth base. BBL phenol broth base with meat extract is also available.

(b) Sterilize 5 or 10% solutions of the carbohydrates, glucosides, or alcohols by filtration through a Seitz or Berkefeld filter.

(c) Add to each tube 0.5 c.c. of the 5% solution, or 0.25 c.c. of the 10% solution of the fermentable substance, with a sterile pipette, under sterile conditions.

M-6. Cystine Trypticase Agar Base.—

Cystine	0.5	gm.
Trypticase	20.0	gm.
Agar	3.5	gm.
Sodium chloride	5.0	gm.
Sodium sulph*ite*	0.5	gm.
Phenol red	0.017	gm.
Dissolve in distilled water	1000	c.c.

The final reaction will be pH 7.3±.

Suspend 29.5 gm. of the BBL powder in 1 liter of distilled water.

Add carbohydrate (0.5 or 1.0 per cent as preferred) if desired. Addition of more than 0.5% carbohydrate may necessitate adjustment of the pH, but with most carbohydrates there will be relatively little change in the reaction.

Mix thoroughly.

Heat gently with frequent agitation.

Boil for about 1 minute, or until solution is complete.

Tube and sterilize at 115° to 118° C., never over 12 pounds pressure, for 15 minutes.

Cool in an upright position and store at room temperature.

Do not refrigerate.

M-7. Serum and Ascitic Fluid Broth With Fermentable Substances.—

Proceed as above (a) to (c). After the fermentable substance has been added, add to each tube 0.5 c.c. of serum or ascitic fluid sterilized by filtration through a Seitz or Berkefeld filter.

Test all tubes for sterility by incubation at 37° C. for 48 hours.

M-8. Nitrate Broth.—

Dissolve 10 gm. tryptone
0.1 gm. potassium nitrate
in 1000 c.c. distilled water.

Adjust the reaction to pH 9.0 to 9.4.

Distribute in 3.5 c.c. amounts in test tubes.

Sterilize in the autoclave for 15 minutes at 15 pounds pressure.

M-9. Tryptose Blood Sodium Azide Broth, Pike.*—

To each 100 c.c. of beef heart infusion broth prepared according to formula M-4 but with 1% tryptose,
add 2 c.c. of 2% sterile solution of dextrose
and 5 c.c. of defibrinated rabbit blood.

Distribute into test tubes, 2 c.c. in each.

For use, add to each tube
0.15 c.c. of 1:1000 solution of sodium azide
0.10 c.c. of 1:25,000 solution of crystal violet.

M-10. Loeffler Blood Serum Medium.—

(a) **Preparation of the blood serum.**

Obtain beef blood in a sterilized, colorless wide-mouth quart bottle.

Allow coagulation to take place without agitation of the container or contents.

Place in the refrigerator until coagulation is complete.

Loosen the clot from the sides of the bottle by means of a sterile stirring rod.

Place in the refrigerator until the serum separates from the clot.

Remove the serum from the clot either with a transfer pipette or by pouring it into a glass container.

Centrifuge the serum in 50 c.c. centrifuge tubes at high speed for fifteen minutes under sterile precautions.

Decant the clear straw-colored supernatant serum, placing it is a glass container.

(b) **Preparation of the medium.**—

Dissolve 1 gm. dextrose
in 100 c.c. of meat infusion broth (formula M-4).

Add 300 c.c. fresh blood serum (cow, sheep, horse, or pig).

Distribute into sterile test tubes.

Slant in the autoclave.

Inspissate and sterilize as directed on page 1395.

Difco-Loeffler blood serum, 80 gm., dissolved in 1000 c.c. distilled water, and BBL-Loeffler's medium, 83 gm., dissolved in 1000 c.c. distilled water, may be used instead of the above medium.

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*Proc. Soc. Exper. Biol. & Med. 57: 186, 1944.

M-11. Ulrich Milk.[*]—

Skim milk	100 c.c.
Methylene blue	0.005 gm.
Chlorphenol red	0.015 gm.
Distilled water	1000 c.c.

Final reaction is pH 6.5±.

Rehydrate BBL or Difco Ulrich milk by the addition of 100 gm. of the powder to a liter of distilled water, preferably preheated to 50° C.

Sterilize at 113° to 115° C. for 20 minutes, or by fractional steam sterilization for 30 minutes on 3 successive days. Avoid overheating and the consequent caramelization which readily occurs.

The uninoculated medium is bluish-gray. The color reactions in inoculated media vary with the amount of peptonization, degree of acidity, or reduction, and other changes produced by the growing organisms, but in general they are as follows:

Uninoculated medium	bluish-gray
Acid	pale yellow-green to yellowish-orange
Alkaline	pale bluish-purple to reddish-purple
Oxidation	bluish-green
Reduction	white
Alkaline, with peptonization	transparent reddish-purple

M-12. Chopped Meat Medium, Robertson.—

Beef heart, Difco	454 gm.
Proteose-peptone No. 3	20 gm.
Dextrose	2 gm.
Sodium chloride	5 gm.

This is **Bacto-Cooked Meat Medium.** Suspend 12.5 gm. of the desiccated product in 100 c.c. of cold distilled water.

After 15 minutes distribute, under constant shaking, into large test tubes, so that each tube will be filled to one-third with meat and to two-thirds with the supernatant fluid.

Sterilize in an autoclave for 15 minutes at 15 pounds pressure.

If the tubes are not used the same day, they should be heated in a boiling water bath for a few minutes shortly before use.

The final pH of the sterilized medium should be 7.2.

M-13. Meat Extract Agar.—

According to the concentration of agar desired, that is, 1.5%, or 2%, soak 15 or 20 gm. agar in 1000 c.c. extract broth (formula M-2) for 15 minutes.

Dissolve the agar *either* by careful heating over a flame *or* on an electric plate, *or* by autoclaving.

Adjust the reaction to pH 7.4 to 7.6

Dispense in tubes or bottles, as desired.

Sterilize for 25 minutes at 15 pounds pressure.

Difco or BBL nutrient agar, 31 gm. of the desiccated medium dissolved in 1000 c.c. of distilled water, is the same as the above formula.

For water analysis, use without sodium chloride.

M-14. Yeast Blood Agar, D'Anniello.—

(a) Yeast extract.—

Dissolve 10 gm. dehydrated yeast in 100 c.c. warm distilled water, stirring with a glass rod.

When dissolved, boil for 10 minutes, no longer.

Filter through gauze and cotton.

Place in a refrigerator for 48 hours.

Decant the supernatant fluid and sterilize by filtration through a Seitz filter.

Store in a refrigerator.

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[*] Science 99: 352, 1944.

(b) **Agar Base.**—

Dissolve Difco blood agar base ---------- 40 gm.
Bacto agar ---------- 5 gm.
in 100 c.c. distilled water by heating.

Distribute into flasks, 500 c.c. to each flask.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

(c) **Medium.**—

Melt 500 c.c. agar base and cool to 45° C.

Add, with sterile precautions, 50 c.c. of yeast extract and 35 c.c. defibrinated blood.

Pour in plates.

M-15. Meat Infusion Agar.—

Infusion broth, double strength, formula M-4	500 c.c.
Agar	15 to 20 gm.
Distilled water	500 c.c.

Dissolve the agar in the distilled water by autoclaving *or* by careful heating. Mix the agar solution and infusion.

Readjust the reaction to pH 7.0.

Distribute into tubes or flasks.

Sterilize in the autoclave at 15 pounds pressure for 20 minutes.

M-16. Tryptose Agar, Difco.—

This contains 20 gm. tryptose, 1 gm. dextrose, 5 gm. sodium chloride, and 15 gm. Bacto-agar for each 1000 c.c.

Dissolve 41 gm. of the desiccated medium in 1000 c.c. of distilled water, distribute in tubes, and sterilize in the autoclave for 20 minutes at 15 pounds pressure.

M-17. Trypticase Agar (Eugonagar*).—

Trypticase	15.0 gm.
Phytone	5.0 gm.
Sodium chloride	4.0 gm.
Sodium citrate	1.0 gm.
Sodium sulphite	0.2 gm.
l-Cystine	0.2 gm.
Dextrose	5.0 gm.
Agar	15.0 gm.
Distilled water	1000 c.c.

Final reaction will be pH 7.0 ±.

Suspend 45.4 gm. BBL Eugonagar, dehydrated, in 1 liter of distilled water.

Allow the mixture to stand for about 5 minutes, and mix until the suspension is uniform.

Heat gently with occasional agitation, and boil for 1 or 2 minutes, or until solution has occurred.

Dispense and sterilize at 118° C. (12 pounds steam pressure) for 15 minutes.

M-18. Trypticase Soy Agar.—

Trypticase	15 gm.
Phytone	5 gm.
Sodium chloride	5 gm.
Agar	15 gm.

Final reaction should be pH 7.3 ±.

Suspend 40 gm. of the BBL powder in a liter of distilled water.

Allow to soak for 5 minutes and mix thoroughly.

Heat gently, with occasional agitation.

Boil for a minute or two or until solution is complete.

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*Vera: J. Bact. 54: 14, 1947.

Dispense, and sterilize at 118° to 121° C. (15 pounds pressure) for 15 minutes when test tubes are used. If large volumes are used, the time but not the temperature may be increased.

For the preparation of blood plates for hemolysis studies, add 5% defibrinated blood to sterile trypticase soy agar which has previously been melted and cooled to about 45° C.

M-19. Dextrose Agar.—

(a) Sterilize 20% solution of dextrose by filtration.

(b) Melt sterile tryptose agar, M-16, or heart infusion agar, M-15.

(c) Add to 100 c.c. of melted agar, 0.5 c.c. dextrose solution (a), and mix. Pour in plates.

M-20. Penicillinase Media.—

To each 5 c.c. of trypticase soy broth (formula M-3), tryptose citrate broth (formula M-24), brain heart infusion broth (formula M-4), or other liquids used for blood cultures, add, with sterile precautions, just prior to inoculation, 0.1 c.c. of 1:100 solution of penicillinase Schenley or Difco. Do not reheat or store.

M-21. Sodium Azide Agar, Snyder and Lichtenstein.*—

Add to each 100 c.c. of blood agar (formulas M-28 or M-29), before pouring, 2 c.c. of 1% solution of sodium azide in distilled water.

Difco sodium azide blood agar base may be used.

M-22. Sodium Azide-Chloral Hydrate Blood Agar.—

(a) Prepare a 1% watery solution of sodium azide and sterilize by filtration through a Seitz filter.

(b) Prepare a 0.1% watery solution of chloral hydrate and sterilize by filtration.

(c) Prepare yeast blood agar as indicated under M-14, but just before pouring plates add to each 500 c.c.

10 c.c. of the sodium azide solution (a)
1 c.c. of the chloral hydrate solution (b).

(d) Pour plates.

M-23. Phenylethyl Alcohol Medium.—

Trypticase	15 gm.
Phytone	5 gm.
Sodium chloride	5 gm.
β-Phenylethyl alcohol	2.5 gm.
Agar	15 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.3 ±.

Suspend 42.5 gm. of the BBL dehydrated product in a liter of distilled water.

Allow to stand for 5 minutes and mix thoroughly.

Heat gently with occasional agitation.

Boil for 1 to 2 minutes or until solution is complete.

Dispense, and sterilize at 118° to 121° C. (12 to 15 pounds pressure) for 15 minutes.

To prepare blood plates, add 5% defibrinated blood to sterile phenylethyl alcohol medium which has been previously melted and cooled to about 45° C.

This medium is not to be depended upon for determination of hemolytic reactions, since atypical reactions may be observed. The usual blood agar plates may be made in parallel to determine the degree of inhibition provided by phenylethyl alcohol medium, and for determination of hemolysis.

A "brown-sugar" appearance is characteristic of the dehydrated product, and is not, therefore, an indication of deterioration.

M-24. Tryptose Citrate Broth, Huddleson.—

Dissolve 20 gm. tryptose
5 gm. sodium chloride
1 gm. dextrose
5 mg. thiamine
10 gm. sodium citrate crystals
in 1000 c.c. distilled water.

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*J. Infect. Dis. 67: 113, 1940; 68: 113, 1941.

Adjust the reaction to pH 7.0.

Fill 20 c.c. amounts in 50 c.c. vaccine bottles.

Insert cotton plugs.

Sterilize in an autoclave for 20 minutes at 15 pounds pressure.

Exchange cotton plugs for sterile rubber diaphragm stoppers.

M-25. Castãneda Medium.*—

(a)	Tryptose	20 gm.
	Sodium chloride	5 gm.
	Sodium citrate	5 gm.
	Agar	30 gm.

Dissolve in 1000 c.c. distilled water by heating.

Put into 100 c.c. flat bottles, 15 c.c. per bottle.

Sterilize in an autoclave for 15 minutes at 15 pounds pressure.

Allow to solidify, laying the bottles on the flat side.

(b)	Tryptose	20 gm.
	Sodium citrate	20 gm.

Dissolve in 1000 c.c. distilled water.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

(c) When the surface of agar (a) is hard, cover it with about 10 c.c. of the tryptose-citrate solution (b).

(d) Exchange the cotton plugs of the bottles for sterile rubber caps and test for sterility after incubating for 24 hours at 37° C.

M-26. Liver Infusion Agar.—

Liver infusion, double strength, formula M-4	500 c.c.
Tryptose or trypticase	10 gm.
Agar	20 gm.
Distilled water	500 c.c.

Dissolve the ingredients in distilled water by heating.

Add the liver infusion.

Filter through glass wool.

Adjust the reaction to pH 7.0

Keep in boiling water bath for 20 minutes.

Filter again.

Adjust the reaction to pH 7.0

Distribute in flasks and sterilize in the autoclave for 30 minutes at 15 pounds pressure.

Bacto-liver infusion agar, 55 gm. per 1000 c.c., is an easily prepared substitute for the above formula.

M-26a. Liver Infusion Agar With Dyes, Huddleson.—

Prepare liver infusion agar (formula M-26), final pH 6.6 to 6.8.

(a) **Crystal Violet Plates.**—

Sterilize 1:700 solution of crystal violet in distilled water by steaming 1 hour.

To each 100 c.c. of the melted and sterile liver infusion agar and 0.1 c.c. of crystal violet solution. Pour in plates.

(b) **Basic Fuchsin Plates.**—

Weigh 1 gm. basic fuchsin and transfer to an Erlenmeyer flask.

Add 100 c.c. sterile distilled water.

Keep in boiling water bath for 20 minutes.

Add 0.4 c.c. of hot basic fuchsin solution to each 100 c.c. of the melted liver infusion agar.

Pour in Petri plates.

(c) **Thionin Plates.**—

Prepare 1% thionin solution just like the basic fuchsin solution above.

To each 100 c.c. of the melted liver infusion agar add 0.2 c.c. thionin solution.

Pour in plates.

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*Proc. Soc. Exper. Biol. & Med. 64: 114, 1947.

M-27. Blood Agar Base.—

This medium is identical with the foregoing formula M-15 except that a peptone such as tryptose, thiotone, or trypticase, more favorable for the growth of fastidious organisms, is substituted for the simple peptone.

Difco-blood agar base with tryptose and BBL-blood agar base with thiotone, 40 gm. of the desiccated powder dissolved in 1000 c.c. distilled water, are excellent for this purpose.

M-28. Blood Agar. Method No. 1.—

Blood agar is a mixture of sterile blood in sterile blood agar base (formula M-27). As it cannot be sterilized after it is made, it is necessary to use the utmost care in preparing it.

For blood agar, rabbit's blood is preferable to human blood, as it does not inhibit the growth of pathogenic bacteria to the same extent as human blood. The blood must be taken in a sterile manner and must be kept sterile throughout the entire procedure. The agar to be used should be blood agar base (formula M-27) previously made and sterilized. It should be liquefied and kept at a temperature between 47° and 50° C. to prevent hemolysis of the blood cells that are to be added.

The medium may be made either in sterile Petri dishes or in sterile test tubes.

(a) Procuring the blood.

Assemble the following:

Rabbit and rabbit board.
Necessary equipment for ether anesthesia.
5 c.c. Luer syringe with a 21 gauge, 1½-inch needle previously sterilized in the dry heat sterilizer at 170° C. two hours.
A bottle, 100 c.c. wide mouth, to which have been added 10 drops of 20% solution of neutral potassium or sodium oxalate. The bottle and contents have been sterilized in the dry heat sterilizer at 170° C. for two hours. The sodium oxalate remains as a fine white powder in the bottom of the bottle.
Tincture of iodine.
Sterile sponges.
Alcohol to remove the iodine.
Bunsen burner.

Place the animal on the animal board.

Shave the skin over heart area.

Give the animal a light anesthetic.

Locate the heart by palpation.

Place a generous quantity of iodine on the skin over the area of the heart to sterilize the surface.

Plunge the needle attached to the syringe into the heart and withdraw 5 c.c. of blood.

Flame the mouth of the bottle containing the sodium oxalate.

Eject the contents of the syringe into the bottle, flaming the needle before ejecting the blood. Replace the cork.

Shake the bottle vigorously to oxalate the blood.

Remove the iodine from the animal's chest with alcohol, and release from board.

(b) Preparing the agar.

Sterile blood agar base (formula M-27) in tubes should be liquefied and kept at a temperature between 47° and 50° C. If blood agar is to be made in slants, there should be 5 c.c. agar in each tube; if it is to be made in 75 mm. Petri dishes, use tubes containing 10 c.c. agar; if using 100 mm. Petri dishes, use tubes containing 15 c.c. agar base.

(c) To make blood agar in Petri dishes.

Place in the center of a sterile Petri dish 0.5 c.c. of the oxalated blood, using a sterile pipette to measure the blood. If 100 mm. Petri dishes are used, use 0.75 c.c. of blood. Care should be taken to exclude all possibility of air contamination during this procedure.

Add to the Petri dish enough liquefied agar (temperature 47° to 50° C.) to fill the plate. Mix thoroughly by rotating the plate. Be sure that an even mixture is obtained. Bubbles are undesirable, because they obscure colony study.

Allow to solidify.

(d) **To make blood agar in test tubes.**

The blood agar base (formula M-27) should be in 5 c.c. quantities in test tubes. Liquefy and keep at a temperature of 47° to 50° C. in a water-bath. Using sterile precautions, add 0.25 c.c. of the oxalated blood to each test tube by means of a sterilized graduated 1 c.c. pipette.

Replace the cotton plug, and rotate the tube vigorously between the palms of the hands.

Slant immediately and allow to solidify.

Place in the incubator at 37° C. for three days for sterility test.

M-29. Blood Agar. Alternate Formula.—

Melt blood agar base (formula M-27), and when cooled to 45° C. add to each 100 c.c. of the medium 5 c.c. of sterile defibrinated human, rabbit, or horse blood.

If the base is kept in tubes each containing 15 c.c. agar, melt the agar by placing tubes in the water bath and add to each tube, when cooled to 45° C., 0.75 c.c. blood using a sterile syringe.

Pour into sterile Petri dishes.

Before solidification, flame the surface of the plates with a Bunsen burner, to free the plate of air bubbles.

M-30. Blood Dextrose Cystine Agar, Francis.—

Dissolve 0.1 gm. cystine and
1.0 gm. dextrose in
100 c.c. of agar (formula M-15) in flowing steam for 1 hour.

Cool to 45° C. and add 7 to 8 c.c. of defibrinated rabbit blood.

Keep in water bath at 60° C. for 2 hours.

Pour in plates or dispense under sterile conditions into sterile test tubes.

Place in incubator for 24 hours to test sterility.

Difco-cystine heart agar, 102 gm. dissolved in 1,000 c.c. distilled water, duplicates the above formula.

M-31. Chocolate Agar.—

Melt blood agar base (formula M-27) and when cooled to 45° C. add to each 100 c.c. of the medium 5 c.c. of sterile defibrinated blood.

If the base is kept in tubes each containing 15 c.c. agar, melt the agar by placing tubes in the water bath and add to each, when cooled to 45° C., 0.75 c.c. of blood, using a sterile syringe.

Put the flask or tubes in a container of warm water. Raise the temperature very slowly to 95° C.

Pour into sterile Petri dishes, or slant the tubes.

M-32. Hemoglobin Chocolate Agar.—

Proteose-peptone No. 3, Difco	20.0 gm.
Bacto-dextrose	0.5 gm.
Sodium chloride	5.0 gm.
Disodium phosphate	5.0 gm.
Bacto-agar	15.0 gm.

Suspend 9 gm. in 100 c.c. of cold distilled water. Dissolve by steaming for a few minutes. Mix well and place in the autoclave for 15 to 20 minutes at 15 pounds pressure.

Dissolve 2 gm. Bacto-hemoglobin in 100 c.c. distilled water at 50° C.

When solution is nearly complete, filter through coarse moistened cheesecloth to remove undissolved particles.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

Cool both this sterile double strength agar and sterile solution of Bacto-hemoglobin to 50 to 60° C.

Mix in equal quantities under sterile conditions, avoiding air bubbles, and pour in sterile Petri dishes.

A satisfactory chocolate agar may also be prepared by adding 5 c.c. of fresh sterile defibrinated blood to 100 c.c. of sterile single strength proteose No. 3 agar at 80° C.

The single strength medium, without enrichment, is prepared by suspending 45 gm. Bacto-proteose No. 3 agar in 1000 c.c. cold distilled water.

Dissolve the medium by boiling for a minute or two. Distribute as desired. Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

The final reaction of the medium without enrichment should be pH 7.3.

M-33. Chocolate Agar With Supplement.—

Supplement A is a yeast concentrate with 1:6,000 crystal violet.

Suspend 22.5 gm. of Bacto-proteose No. 3 agar in 250 c.c. distilled water. Dissolve by boiling or holding in a boiling water bath.

Sterilize in the autoclave for 15 to 20 minutes at 15 pounds pressure.

Suspend 5 gm. Bacto-hemoglobin in 2500 c.c. distilled water, below 50° C. Shake well to obtain an even suspension. Sterilize in the autoclave for 15 to 20 minutes at 15 pounds pressure (121° C.).

Cool the sterile double strength agar and 2% hemoglobin solution to 50 to 60° C.

Mix under sterile conditions.

Add with sterile precautions 5 c.c. of Bacto-supplement A, and mix well. Pour into Petri dishes.

M-34. Hirschberg Transport Medium.*—

Dissolve 4.5 gm. proteose No. 3 agar, Difco
4.0 gm. gelatin
0.6 c.c. of 0.04% Nile blue A
0.5 gm. agar
in 80.0 c.c. distilled water.

Adjust the reaction to pH 7.4.

Sterilize in the autoclave for 15 minutes at 15 pounds pressure.

Cool to 50° C. and add 20 c.c. of sterile defibrinated horse blood. Heat to 80 to 95° C. until chocolate agar is formed.

M-35. McLeod-Sanders-Hullinghorst Medium.†—

(a) Bacto-proteose No. 3 agar	45 gm.
Agar	5 gm.

Dissolve in 1000 c.c. distilled water by heating.

Sterilize in an autoclave at 15 pounds pressure for 20 minutes.

(b) 10% yeast extract (*see* Yeast Agar M-14).

(c) Before use, melt agar (a) and add to each 100 c.c.

Yeast extract (b)	10 c.c.
Human blood	7.5 c.c.

This medium may be heated to 80° C. and kept at that temperature until chocolatized.

(d) Pour plates.

M-36. Carpenter Medium No. II.‡—

(a) Proteose-peptone No. 3	15 gm.
Sodium chloride	5 gm.
Dipotassium phosphate (K_2HPO_4)	4 gm.
Monopotassium phosphate (KH_2PO_4)	1 gm.
Agar	20 gm.
Cornstarch	1 gm.

Dissolve in 1000 c.c. distilled water by heating.

This medium is available in desiccated form from Difco, Inc., under the name Bacto G.C. Medium.

Place in 100 c.c. quantities in 500 c.c. flasks, and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

(b) Agitate 10 gm. Bacto-hemoglobin in 500 c.c. distilled water for 10 minutes or longer, then filter through gauze to remove undissolved particles.

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*South. M. J. 38: 493, 1945.

†Am. J. Pub. Health 41: 10, 1951.

‡Am. J. Syph. 33: 163, 1949.

Dispense in 100 c.c. amounts and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

(c) Before use, melt base (a), and add an equal amount of hemoglobin solution (b), and 1% Difco Supplement B.

(d) Pour plates.

M-37. Hormone-Brom Cresol Purple Agar, Gradwohl.—

(a) **Preparation of Ascitic Fluid for Use in Cultivation of the Gonococcus.**—Prepare a large (at least 2 quarts) colorless bottle, stoppered with a cork, by sterilizing it in the dry heat sterilizer.

Request the physician who is to collect the ascitic fluid not to place his finger over the end of the trocar, and to hold sterile paper or gauze over the bottle as the ascitic fluid is being collected. Use all aseptic precautions.

Place the ascitic fluid in the refrigerator until the sterility test has been completed.

Inoculate 3 tubes of M-47 and 3 of M-48, using the top of the fluid, fluid from the center, and from the bottom of the bottle. Place the ascitic fluid back in the refrigerator.

Place the 3 tubes in the incubator at 37° C. for 3 to 10 days. If no growth occurs in this time, the ascitic fluid is sterile and may be ampuled. If it is not sterile, it must be discarded.

If it is sterile, ampule, seal hermetically, and store in a refrigerator.

(b) **Preparation of the Base.**—Infuse 500 grams of lean chopped beef in 500 c.c. of distilled water in the refrigerator overnight.

The following morning, heat to 60° C.

Dissolve 20 grams agar in 500 c.c. distilled water by boiling.

Add the melted agar to the heated beef infusion, and bring to a vigorous boil.

Press through a wire gauze strainer. The filtrate will be very cloudy.

Add 20 grams Merck's peptone and 5 grams sodium chloride.

Heat to dissolve.

Adjust the reaction to pH 7.8-8.2.

Boil vigorously for about ten minutes to precipitate the phosphates.

Cool to 60° C. Dilute to volume with distilled water.

Stir in a mixture of one egg yolk in 10 c.c. distilled water.

Boil vigorously for fifteen minutes to coagulate the egg.

Allow to stand until flocculence of the egg has occurred.

Filter through glass or steel wool, carrying out the filtration in an Arnold steam sterilizer at a temperature just hot enough to keep the agar liquid. The filtrate should be crystal clear. Pour the filtrate back through the funnel if it is cloudy. This may have to be repeated several times.

To every 100 c.c. clarified agar add 1 gram levulose, c.p., and 2.5 c.c. 0.5% aqueous solution of brom cresol purple indicator.

Mix thoroughly.

Place in 10 c.c. quantities in test tubes.

Plug with nonabsorbent cotton.

Sterilize in the autoclave at 7 to 10 pounds pressure for forty-five minutes.

Place in the incubator for three days for sterility test before using.

Note.—If the medium is made in large quantities it keeps better in screw-cap vials than in cotton-plugged test tubes.

For use, melt a tube of the base (b) and cool to 45° C.

Place 2 to 3 c.c. ascitic fluid (a) in a sterile Petri dish, 75 mm. diameter.

Pour the base into the plate and mix by rotating the dish.

M-38. Glycerin Potato Veal Agar.—

Boil sliced potatoes with double volume of water. Filter through cotton.

To each 100 c.c. of filtrate add 40 c.c. glycerin.

Place in the autoclave for 30 minutes at 15 pounds pressure.

Melt 100 c.c. veal infusion nutrient agar (formula M-15).

Add 5 c.c. sterile potato glycerin mixture.

Pour into plates. Do not use plates for 24 hours after pouring.

M-39. Potato Glycerin Blood Agar, Bordet and Gengou.—

Potatoes, peeled and mashed	100	gm.
Glycerin	8	c.c.
Proteose-peptone	5	gm.
Sodium chloride	2.25	gm.
Agar	10	gm.
Distilled water	500	c.c.

Mix the potatoes and the glycerin with 200 c.c. distilled water. Boil, and when soft, dilute to volume with distilled water.

Strain through gauze.

Allow to settle.

Use the clear supernatant fluid.

To 100 c.c. of the supernatant fluid add the other ingredients, and the rest of the distilled water (300 c.c.).

Let soak for 15 minutes.

Dissolve by boiling.

Adjust the reaction to pH 6.4 to 6.6.

Distribute in flasks and sterilize in the autoclave for 25 minutes at 15 pounds pressure.

Before use, melt 100 c.c. of the base, let cool to 45° C., and add 20 c.c. of fresh human, rabbit, horse, or sheep blood.

Pour into plates.

The base may be purchased as Difco or BBL Bordet-Gengou Agar Base. Dissolve 40 gm. in 1000 c.c. of 1% glycerin in water as a substitute for the above formula.

M-40. Penicillin Agar, Bradford.*—

Add to medium M-39 (or to any other blood agar medium), just before pouring, 0.2 to 0.25 unit of penicillin for each c.c. of medium.

M-41. Tellurite Medium, Difco.—

Proteose-peptone No. 3	20	gm.
Bacto-dextrose	2	gm.
Sodium chloride	2	gm.
Agar	13	gm.

Suspend 40 gm. of Bacto-dextrose proteose No. 3 agar in 1000 c.c. cold distilled water. Boil for a minute or two to dissolve the medium, and distribute as desired. Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

Add 5 c.c. Bacto-tellurite blood solution to each 100 c.c. of sterile melted agar at 70 to 80° C. under sterile conditions.

Heat at 75 to 80° C. until it has the appearance of a ''chocolate'' agar and then cool to 50° C.

Distribute into sterile tubes or plates as desired.

Final reaction of the medium without enrichment is pH 7.4.

M-42. Tellurite Agar, BBL.—

Trypticase	20	gm.
Sodium chloride	5	gm.
Dextrose	2	gm.
Agar	20	gm.
Distilled water	1000	c.c.

The final reaction should be pH 7.5 ±.

Suspend 47 gm. of the BBL powder in a liter of distilled water.

Mix thoroughly until a uniform suspension is obtained.

Heat gently, with frequent agitation.

Boil for 1 or 2 minutes until solution is complete.

Sterilize at 118° to 121° C. (12 to 15 pounds pressure) for 15 minutes.

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*Am. J. Pub. Health 36: 468, 1946.

To the sterile fluid agar, cooled to a temperature of about 50° C., add

Sterile serum (e.g., human)	50 c.c.
1% Tellurite, pH 9.0 to 9.5	5 or 10 c.c.

to each 1000 c.c. of medium.

Mix and pour into plates, using at least 20 c.c. of the complete medium in each plate. Plates so prepared should be usable for about 2 weeks if stored in a refrigerator.

If preferred, whole blood may be used instead of serum, and the tellurite concentration may be increased. However, colonial types may be less readily distinguished on the medium with blood, and inhibition of growth of some strains of Corynebacterium may occur when a higher tellurite content is employed.

M-43. Whitney-Damon Medium.*—

(a) Buffered water.—

M/15 disodium phosphate (Na_2HPO_4)	72 c.c.
M/15 monosodium phosphate (NaH_2PO_4)	28 c.c.
Distilled water	900 c.c.

Keep several bottles in stock.

(b) Tellurite solution.—

Grind 0.5 gm. potassium tellurite with 10 c.c. portions of buffer (a); pipette off and collect the supernatant fluid. A few drops of 10% potassium hydroxide may be added to facilitate solution. The final solution must be 0.5%. Sterilize by filtration through a Seitz filter.

(c) 10% watery solution of raffinose. Sterilize by Seitz filtration.

(d) Dissolve Difco-proteose No. 3 agar	45 gm.
in buffer solution (a)	1000 c.c.

Place in an autoclave at 15 pounds pressure for 15 minutes.

(e) Before use melt agar (d).

(f) Prepare a mixture of

Sterile human serum	60 c.c.
Raffinose solution (c)	48 c.c.
Tellurite solution (b)	12 c.c.

(g) Add 10 c.c. of mixture (f) to each 100 c.c. of base (d).

(h) Pour plates.

M-44. Elek, King, et al., Medium.†—

Proteose-peptone	20 gm.
Agar	15 gm.
Sodium chloride	2.5 gm.

Dissolve in 1000 c.c. distilled water.

Adjust the reaction to pH 7.8.

Place in 10 c.c. quantities in test tubes, and sterilize in an autoclave.

Before use, melt and add to each tube 2 c.c. of fresh, nonhemolytic rabbit or horse serum.

M-45. Phenol Red Mannitol Salt Medium, Chapman.—

Mannitol	10 gm.
Beef extract	1 gm.
Proteose-peptone No. 3, *or* Polypeptone	10 gm.
Sodium chloride	75 gm.
Agar	15 gm.
Phenol red	0.025 gm.

Suspend 111 gm. Difco or BBL phenol red mannitol salt agar in 1000 c.c. cold distilled water. Boil for 1 or 2 minutes to dissolve the medium.

Dispense, and sterilize in an autoclave for not more than 15 minutes at 15 pounds pressure.

The final reaction will be pH 7.4.

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*Pub. Health Rep. **64**: 457, 1949.

†Am. J. Pub. Health **39**: 1314, 1949.

M-46. Chapman-Stone Medium.—

Yeast extract	2 to 2.5 gm.
Trypticase *or* Tryptone	10 gm.
Gelatin	30 gm.
d-Mannitol	10 gm.
Sodium chloride	55 gm.
Ammonium sulphate	75 gm.
Dipotassium phosphate	5 gm.
Agar	15 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.0 ±.

Suspend 20.2 gm. of the BBL or Difco dehydrated material in 100 c.c. of distilled water.

Allow to stand for 5 minutes.

Mix to obtain a uniform suspension.

Heat and boil for 1 or 2 minutes or until solution is complete.

Pour into plates while hot, being careful to avoid the formation of bubbles.

After solidification, the medium should be white and opaque.

If the medium cannot be used on the day of preparation, it should be dispensed and sterilized by autoclaving at 121° C. (15 pounds pressure) for 15 minutes.

M-47. Thioglycollate Medium, Brewer-Vera.—

To 750 c.c. of 2% polypeptone in water

Add 6 gm. dextrose
2.5 gm. sodium chloride
0.25 gm. l-cystine
0.1 gm. sodium sulph*ite*
0.5 gm. sodium thioglycollate
0.7 gm. agar dissolved in 250 c.c. distilled water.

Fill high columns of the liquid into large test tubes.

Sterilize in an autoclave for 20 minutes at 15 pounds pressure.

Store at room temperature.

Before use, heat to 80° C. for a few minutes and let cool.

This medium may be purchased in dehydrated form from Baltimore Biological Laboratories as thioglycollate medium. Dissolve 30 gm. in 1000 c.c. distilled water.

M-48. Fluid Thioglycollate Medium.—

Trypticase	15	gm.
l-Cystine	0.75	gm.
Dextrose	5	gm.
Yeast extract	5	gm.
Sodium chloride	2.5	gm.
Sodium thioglycollate	0.5	gm.
Resazurin	0.001	gm.
Agar	0.75	gm.

The Difco product contains casitone instead of trypticase and 0.3 c.c. thioglycollic acid instead of the sodium salt.

Suspend 29.5 gm. of the BBL or Difco powder in 1000 c.c. distilled water.

Allow to soak 5 to 10 minutes.

Mix thoroughly and then heat with occasional agitation of the medium.

Boil until solution is complete. The reaction must be pH 7.1.

Dispense in test tubes, filling them half full, using about 15 c.c. in 6 by ¾ inch tubes. If flasks are preferred, the ratio of surface to volume should be similar to that of tubes.

For maintenance of cultures, place a small quantity (approximately 0.1 gm.) of calcium carbonate ($CaCO_3$) in each tube before introducing the liquid.

Sterilize in the autoclave for 15 to 18 minutes at 15 pounds pressure.

Cool before use.

Store at room temperature, not in the refrigerator.

M-49. Thioglycollate Agar, Brewer.—

(a)	Polypeptone	20 gm.
	Sodium chloride	2.5 gm.
	Dextrose	10 gm.
	Agar	15 gm.
	l-Cystine	0.4 gm.
	Sodium thioglycollate	2 gm.
	Sodium formaldehydesulfoxylate	1 gm.
	Methylene blue, certified	0.002 gm.

Final reaction should be pH 7.2.

Preferably use the desiccated form produced by Baltimore Biological Laboratory. Use 51 gm. per 1000 c.c.

(b)	Trypticase	20 gm.
	Sodium chloride	5 gm.
	Agar	15 gm.
	Sodium thioglycollate	2 gm.
	Sodium formaldehydesulfoxylate	1 gm.

Final reaction should be pH 7.2.

Use 43 gm. per 1000 c.c.

After melting and sterilizing, add 1.5 to 5% blood.

Thioglycollate media should not be sterilized longer than 20 minutes at 15 pounds pressure.

M-50. Anaerobic Nitrate Broth, Reed and Orr.—

Dissolve 10 gm. tryptose
5 gm. sodium chloride
1 gm. potassium nitrate
1 gm. agar
3 gm. meat extract
1 gm. sodium thioglycollate
in 1000 c.c. distilled water, with heat. Filter while hot.

Adjust the reaction to pH 7.0.

Add 0.001 gm. Resazurin.

Distribute into test tubes and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Check for sterility.

Difco anaerobic nitrate broth and BBL indol nitrate broth, 25 gm. dissolved in 1000 c.c. distilled water, have similar formulas.

M-51. Iron Milk, Reed and Orr.—

Dissolve 100 gm. Difco skim milk with brom cresol purple
in 1000 c.c. distilled water.

Strain and distribute in 10 c.c. amounts in test tubes.

Add 1 gm. reduced iron* to each tube. Sterilize in the autoclave at 15 pounds pressure for 30 minutes.

Nails can be used and re-used after boiling in 50% hydrochloric acid and rinsing in tap water.

M-52. Iron-Gelatin for Anaerobes.—

Dissolve 120 gm. gelatin
8 gm. nutrient broth, desiccated, BBL or Difco
1 gm. dextrose
in 1000 c.c. distilled water.

Adjust the reaction to pH 7.0 to 7.2.

Distribute in test tubes.

Put 1 washed iron nail (see above) in each tube.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

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*See formula M-52.

M-53. Motility Thioglycollate Medium.—

Thioglycollate broth, dehydrated, BBL or Difco	29.5 gm.
Agar	4.0 gm.

Dissolve in 1000 c.c. distilled water.

Tube and place in an autoclave at 15 pounds pressure for 15 minutes.

M-54. Thioglycollate Base.—

Trypticase	20 gm.
Sodium chloride	5 gm.
Dipotassium phosphate	2 gm.
Sodium thioglycollate	1 gm.
Agar	0.5 gm.
Distilled water	1000 c.c.

Final reaction should be pH 7.2 ±.

Suspend 28.5 gm. of the BBL powder in a liter of distilled water.

Allow to soak about 5 minutes.

Mix thoroughly.

When the suspension is uniform, heat gently, with occasional agitation, until the medium boils for 1 or 2 minutes and becomes clear.

Add 5 to 10 gm. of carbohydrate, if the medium is to be used for fermentation studies or for diagnostic work.

Dispense in test tubes, filling them half full, and using about 15 c.c. in 6 by ¾ inch tubes.

Sterilize by autoclaving at 118° to 121° C. (12 to 15 pounds pressure) for 15 minutes.

Add indicator after incubation, to detect decomposition of the added carbohydrate.

The medium should not be used until it has cooled to room temperature. The prepared medium should be stored in the dark at room temperature, *not in the refrigerator.* It may be used until not more than one-third of the column of liquid has been oxidized, as shown by the indicator. After prolonged storage, boil the medium for a few minutes and cool before use.

M-55. Thioglycollate Glycerol.—

10% yeast extract (*see* M-14)	50 c.c.
Proteose-peptone No. 4	15 gm.
Sodium chloride	2.5 gm.
d-l-Cystine	0.05 gm.
Sodium thioglycollate	0.5 gm.
Agar	0.75 gm.
Glycerol	20 gm.

Dissolve in 930 c.c. distilled water by heating.

Adjust the reaction to pH 7.4.

Add 0.1% brom cresol purple	7.5 c.c.

Dispense in screw-capped bottles.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

M-56. Trypticase Lactose Iron Agar, BBL.—

Trypticase	20 gm.
Agar	3.5 gm.
Lactose	10 gm.
Ferrous sulphate	0.2 gm.
Sodium sulph*ite*	0.4 gm.
Sodium thiosulphate	0.08 gm.
Phenol red	0.02 gm.

Use 34.2 gm. per liter of solution. Final reaction is pH 7.2. Sterilize in the autoclave for 25 minutes at 10 to 12 pounds pressure.

M-57. Nagler and McClung-Toabe Plate.*—

Proteose-peptone No. 2 or No. 3	40 gm.
Disodium phosphate	5 gm.

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*J. Bact. 53: 2, 1947.

Monopotassium phosphate	1 gm.
Sodium chloride	2 gm.
Magnesium sulphate	0.1 gm.
Dextrose	2 gm.
Agar	25 gm.

Dissolve in 1000 c.c. distilled water by heating.

Adjust the reaction to pH 7.4.

Sterilize in an autoclave at 15 pounds pressure for 20 minutes.

Before use, melt, cool to 45° C., and add to each 90 c.c. of the medium 10 c.c. of sterile egg yolk.

Pour plates.

Sodium azide, 0.02%, may be added.

M-58. Petrik Medium.*—

All glassware and utensils coming in contact with the medium are sterilized in the autoclave for 30 minutes, at 15 pounds pressure.

Ingredients.—

Potato-Extract.—To 1,000 c.c. of 8.4 volume per cent glycerin water add 250 gm. of thinly sliced, freshly peeled potato with a minimum exposure to air. Autoclave with the glassware.

Eggs.—Wash fresh eggs in water, and immerse them in 70% alcohol for two hours.

Malachite-Green Oxalate.—Sterile 2% aqueous solution (2 gm. per 100 c.c. distilled water).

Loewenstein Solution.—

Asparagin	2.4 gm.
Primary potassium phosphate (KH_2PO_4)	1.6 gm.
Magnesium sulphate	0.16 gm.
Distilled water	50.0 c.c.

Autoclave with the glassware.

Potato-Egg Medium. Technic.—

Into a three quart pot, pour 500 c.c. of whole egg, and 100 c.c. of egg yolk.

Beat the mixture thoroughly.

Cool 350 c.c. of potato-extract to about 50° C., and add 12.5 c.c. of 2% aqueous malachite green solution, and the entire quantity (given above) of Loewenstein solution.

Beat thoroughly with an egg beater, and filter through several thicknesses of sterile gauze into the tubing funnel.

Place 10 to 12 c.c. in each tube, stopper the tube with a cotton plug, and slant in the inspissator or Arnold steam sterilizer.

Heat at 85° C. for exactly one-half hour. If the inspissator is used for heating the tubes, stopper them with cork stoppers to prevent loss of water from the medium during inspissation. If the Arnold is used, stopper the tubes soon after they are removed from the sterilizer. One heating is sufficient if sterile precautions are maintained during the preparation of the medium.

Incubate the tubes for three days to test for sterility.

After incubating for sterility, as an aid in keeping the moisture in the medium till ready for use and also to prevent drying out after planting and placing in the incubator, paraffin the stoppers of the tube with hot paraffin. Tubes can then be kept in the incubator for a longer period of time without the loss of a large amount of their moisture.

M-58a. Blood Agar Medium, Tarshis** **(for growth of tubercle bacillus).**—

Glycerin, analytical grade	3 gm.
Distilled water	219 c.c.

Dissolve the glycerin in the distilled water and add

Blood agar base, Difco, desiccated	9 gm.

Dissolve by heating in a boiling water bath.

Autoclave for 15 minutes at 15 pounds pressure.

Cool to 50° C.

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*Ryley, John P.: Lab. Digest, July, 1941, p. 4, and personal communication.

J. Lab. & Clin. Med. **40: 628, 1952.

Add, under sterile precautions:

Human blood	75 c.c.
Penicillin solution, 75 U. per c.c.	3 c.c.

Mix well and keep in a 50° C. water bath while dispensing into sterile screw-capped tubes, about 6 c.c. per tube.

Allow to solidify with long slants.

Test for sterility after incubating for 48 hours.

M-58b. Charcoal Agar Medium, Whalen.*—

Dissolve with the aid of heat:

Proteose peptone No. 3, Difco	2 gm.
Asparagin	1 gm.
Disodium phosphate 12 H_2O	2.5 gm.
Monopotassium phosphate, anhydrous	1 gm.
Magnesium phosphate	0.5 gm.
5% aqueous solution of ferric ammonium citrate	10 c.c.
Sodium citrate	0.5 gm.
0.01% aqueous solution of cobaltous sulphate	1 c.c.
Savita yeast-vegetable extract	2 gm.
Dextrose	7.5 gm.
Glycerin	2.5 c.c.
1% solution of oleic acid in N/20 sodium hydroxide	8 c.c.
0.1% aqueous ethyl violet	3 c.c.
Agar	15.6 gm.

in 1 liter of distilled water.

Add, under thorough mixing, 4 gm. Nuchar C-190-N activater carbon.

Tube in approximately 6 c.c. quantities in screw-capped tubes, while stirring.

Autoclave at 15 pounds pressure for 15 minutes.

Allow to solidify with long slants.

M-58c. Egg Medium, Kauffmann.—

See page 1421 for preparation of whole eggs.

Weigh sterile thick-walled Erlenmeyer flasks containing beads.

Add 8 whole eggs.

Weigh the flask again and subtract the weight of the dry flask from the weight of the flask plus eggs. This gives the weight of eggs.

Add 0.85% sterile saline, the amount equivalent to one-third of the weight of the eggs.

Mix well and filter through sterile gauze. Distribute with sterile precautions into sterile tubes. Slant and inspissate for 1 hour at 80° C. on two consecutive days.

M-59. Egg Medium, Dorset.—

See page 1421 for the preparation of eggs.

Mix, in a sterile flask containing glass beads, 16 whole eggs and 100 c.c. of 0.85% sterile saline.

Mix and filter through sterile gauze.

Tube and sterilize as directed for egg medium M-58.

M-60. Loewenstein-Jensen-Holm Medium, Asparagin-Starch-Egg.—

(a) Asparagin solution.—

Dissolve 2.4 gm. potassium sulphate
0.24 gm. magnesium sulphate
0.6 gm. magnesium citrate
3.6 gm. asparagin
12.0 gm. glycerin
in enough distilled water to make 600 c.c. solution.

Sterilize in flowing steam for 2 hours.

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*Bact. Proc. 55: 98, 1955.

(b) Add 30 gm. potato flour to solution (a).

Keep in water bath under constant stirring until sticky. Then allow to stand in the water bath for 1 hour at 56° C.

(c) Prepare whole eggs as described on page 1421.

Add 1000 c.c. of homogenized whole eggs to solution (b) and mix well.

Add 20 c.c. of 2% solution of malachite green previously sterilized in the autoclave at 15 pounds pressure for 15 minutes.

Mix well. Filter through gauze. Distribute into tubes.

Inspissate the first day ½ hour at 85° C.

Heat again the second day for ½ hour at 75° C.

M-61. Petragnani-Frobisher Medium.—

(a) Mix in an Erlenmeyer flask

Milk	225 c.c.
Potato starch	9 gm.
Peptone	1.5 gm.
Diced potatoes	150 gm.

Immerse the lower part of the flask in boiling water, shaking frequently, until the contents are sticky, then leave the flask in the water bath for 1 hour. Before use, cool to 60° C.

(b) Homogenize, preferably in a Waring blender,

Whole eggs	8
Glycerol	18 c.c.
2% watery malachite green	15 c.c.
(c) Dextrose	1.5 gm.
Asparagin	1.5 gm.

Dissolve in 50 c.c. distilled water by heating.

(d) Mix (a), (b), and (c).

Filter through gauze.

Tube and sterilize as directed for egg medium M-60.

M-62. Dubos Media.*—

Dubos broth base, with and without Tween 80; Dubos oleic agar base; Bacto-TB broth base, with and without Tween 80; Dubos serum; and Dubos albumin are best purchased from Difco, Inc.

For testing sensitivity of *Mycobacterium tuberculosis* to antibiotics and for the Dubos-Middlebrook neutral red virulence test of *M. tuberculosis,* the TB broth base without Tween 80 is recommended.

M-63. MacConkey Plate.—

Dissolve 20 gm. peptone (*or* 17 gm. peptone and 3 gm. proteose-peptone) in 1000 c.c. distilled water.

Add 5 gm. sodium chloride.

Add 5 gm. sodium glycocholate (*or* taurocholate *or* 1.5 gm. Bacto-bile salts No. 3) and 15 to 20 gm. granulated agar (20 is preferable).

Soak for 15 minutes, then dissolve by heating.

Adjust the reaction to pH 7.4.

Add 10 gm. lactose to the warm agar.

Sterilize in the autoclave at 15 pounds pressure for 20 minutes.

Add, with sterile precautions, 3 to 4 c.c. of 1% aqueous neutral red "certified." Boil the neutral red solution in a tube for five minutes before adding it to the medium.

When partly cooled, pour in Petri dishes, about 18 to 20 c.c. per dish. Allow to stand with slightly removed tops for one hour in a dust-free place at room temperature.

Store in the refrigerator.

There are numerous modifications of MacConkey's medium. Those made by BBL and by Difco are excellent; they contain one part of crystal violet for one million parts of the medium. This combination inhibits gram-positive microorganisms.

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*J. Exper. Med. 83: 409. 1946; Am. Rev. Tuberc. 36: 334. 1942.

M-64. Desoxycholate Agar, Leifson.—

Soak 10 gm. peptone
5 gm. sodium chloride
10 gm. lactose
17 to 20 gm. agar
in 1000 c.c. distilled water for 15 minutes.
Dissolve by heating.
While still hot, add quickly and in the following order
2 gm. anhydrous dipotassium phosphate
2 gm. ferric ammonium citrate "green scales"
1 gm. sodium desoxycholate.
Adjust the reaction to pH 7.4.
Add 3 c.c. of 1% aqueous solution of neutral red.
Heat in steam for 15 minutes.
Pour plates in 17 to 20 c.c. quantities.
Allow to solidify with covers partially removed, for about 1 hour.
Store in the refrigerator.

BBL desoxycholate agar, 46 gm. dissolved in 1000 c.c. distilled water, duplicates the above formula.

M-65. Desoxycholate-Citrate Agar, Leifson.—

Infuse 1000 gm. ground lean pork meat
in 3000 c.c. distilled water at room temperature for 3 hours.
Add 10 c.c. of N/1 hydrochloric acid, and boil one minute.
Squeeze through gauze a small amount at a time, then filter through filter paper.
Add to the filtrate 10 c.c. of N/1 sodium hydroxide.
Boil 1 minute and dilute to 3000 c.c. with distilled water.
Add 30 gm. peptone (Thio or Wilson B).
Adjust the reaction to pH 7.5.
Boil for 5 minutes and filter through filter paper.
Add 60 gm. agar
and 15 c.c. N/1 sodium hydroxide.
Allow to soak at room temperature for 15 minutes.
Boil in steam for 20 minutes.
Place in Erlenmeyer flasks in 1000 c.c. quantities (or smaller amounts).
Add 10 gm. lactose
20.6 gm. crystalline sodium citrate
5 gm. sodium desoxycholate (optional 10 c.c. of 0.35% aqueous lead chloride solution)
to each 1000 c.c.
Adjust the reaction to pH 7.4.

Either store in the refrigerator immediately *or* add at once 2 gm. ferric ammonium citrate "green scales" and 3 c.c. of 1% aqueous solution of neutral red.

Pour in plates, 15 to 17 c.c. in each. Allow to dry with partially removed covers for 1½ hours. Store in refrigerator.

Small dewdrop-like crystals of sodium desoxycholate appear on the surface of the medium during storage. Their presence does not influence the quality of the medium. The "drops" disappear when the plate is incubated.

BBL desoxycholate-citrate agar with infusion, 73 gm. dissolved in 1000 c.c. distilled water, duplicates the above formula and saves much work.

M-66. Desoxycholate-Citrate Lactose Sucrose Agar, BBL.—

Sodium desoxycholate	2.5 gm.
Sodium citrate	10.5 gm.
Lactose	5.0 gm.
Sucrose	5.0 gm.
Polypeptone	7.0 gm.

Meat extract	3.0 gm.
Sodium thiosulphate	5.0 gm.
Neutral red	0.03 gm.
Agar	12.0 gm.

Suspend 50 gm. of the powder in 1000 c.c. distilled water. Mix thoroughly so that the suspension is uniform. Heat gently with occasional agitation of the medium. Boil for 1 or 2 minutes or until solution is complete. Cool the medium to about 50° C. and pour into Petri dishes. The plates may be used as soon as they have solidified, or they may be stored in the refrigerator for a few days.

If the surface of the agar appears to be wet, it is possible to dry it rapidly by inverting the plates and then tilting the bottom halves, containing the solid agar, on the edges of the lids.

M-67. S.S.* Agar.—

Beef extract	5 gm.
Proteose-peptone or Polypeptone	5 gm.
Lactose	10 gm.
Bile salts	8.5 gm.
Sodium citrate	8.5 gm.
Sodium thiosulphate	8.5 gm.
Ferric citrate	1 gm.
Agar	13.5 gm.
Brilliant green	0.33 mg.
Neutral red	0.025 gm.

Suspend 60 gm. of the Difco or BBL product in 1000 c.c. distilled water.

Boil for 1 or 2 minutes to dissolve completely.

Do not sterilize in the autoclave.

About 20 c.c. of the medium should be poured into standard Petri dishes 90 to 100 mm. in diameter.

It is very important that the surface of the plates be quite dry when inoculated. This is accomplished by allowing the medium to solidify and to stand for 2 hours with the covers partially removed.

Final reaction is pH 7.0.

M-68. D.E.C.† Agar, Panja and Ghosh.[1]—

Dissolve 5 gm. beef extract (Lemco or other)
5 gm. peptone, Difco
8.5 gm. sodium taurocholate
25 gm. agar
in 1000 c.c. distilled water.

Steam for 1 hour.

Adjust the reaction to pH 7.0.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

For use, melt, and add in order named:

Sodium citrate, crystals	8.0 gm.
Sodium thiosulphate, crystals	8.5 gm.
Disodium phosphate, crystals	7.5 gm.
Lactose	12.5 gm.
Ferric citrate "green scales"	3.0 gm.

Adjust the reaction, if necessary, to pH 7.4.

Add 15 c.c. of 0.25% solution of neutral red

and 5 c.c. of 2N sodium hydroxide.

Boil for 2 minutes.

Pour in plates.

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*Salmonella-Shigella.

†Desoxycholate-Citrate.

[1] Indian. M. Gaz. 78: 55, 1943.

M-69. Bismuth Sulphite Agar, Hajna and Perry.—

(a) **Agar Base.—**

Agar	30 gm.
Beef extract	5 gm.
Peptone	10 gm.
Sodium chloride	5 gm.
Distilled water	1000 c.c.

Dissolve in the autoclave.

Adjust the reaction to pH 7.4 to 7.6.

Sterilize in the autoclave for 30 minutes at 15 pounds pressure and store in the refrigerator.

(b) **Hajna-Perry Reagent.—**

(1) Dissolve 80 gm. anhydrous sodium sulphite, Baker, in 400 c.c. boiling distilled water.

(2) Add 40 c.c. distilled water to 24 gm. bismuth citrate, c.p., mix to a paste, and add at once 10 c.c. pure ammonia (sp. gr. 0.897) and then drop by drop add more ammonia until the mixture is only fluorescent. Dilute to 200 c.c. with distilled water.

Mix the sodium sulphite and the bismuth solutions.

(3) Add 42 gm. of anhydrous dibasic sodium phosphate, Baker, to the above mixture and mix well until nearly dissolved.

(4) Dissolve 3.2 gm. crystalline ferrous sulphate, c.p., in 40 c.c. of distilled water.

Add 2 drops of concentrated hydrochloric acid, and add this solution to the above mixture. Cover the mixture with a beaker and boil carefully until the color turns slate gray. Store in dark, tightly stoppered bottle.

(c) **1% Aqueous Solution of Brilliant Green ''Certified for Media.''—**

(d) **For Use.—**

To 1000 c.c. melted, partly cooled base (a)
add 10 gm. glucose
70 c.c. Hajna-Perry reagent (b)
3 to 4 c.c. brilliant green solution (c).

Pour in plates as for the original Wilson-Blair medium.

M-70. Bismuth Sulphite Plate, Difco and BBL.—

Peptone	10 gm.
Beef extract	5 gm.
Dextrose	5 gm.
Disodium phosphate	4 gm.
Ferrous sulphate	0.3 gm.
Bismuth sulph*ite* indicator	8 gm.
Agar	20 gm.
Brilliant green	0.025 gm.

Suspend 5.2 gm. bismuth sulphite agar in 100 c.c. cold distilled water. Bring to a boil as rapidly as possible and allow it to simmer for 1 to 2 minutes.

If the medium is prepared several days before use, it should be poured into plates and stored in a cold moist atmosphere to prevent drying. The melted medium should never be allowed to solidify in flasks and be remelted.

Final reaction of the medium is pH 7.7.

M-71. Eosin Methylene Blue Agar, Holt-Harris and Teague.—

Use nutrient agar, 2%, made with 0.3% beef extract (3 gm. per 1000 c.c.), formula M-13.

To 1000 c.c. of melted agar base, add
50 c.c. 10% aqueous sterile solution of lactose
(optional: 10% sterile sucrose, 50 c.c.)
20 c.c. of 2% aqueous solution of eosin Y
and 20 c.c. of 0.5% to 0.65% aqueous methylene blue solution.

Pour in plates.

Dry with lids closed.
Store in the refrigerator in inverted position.
BBL and Difco desiccated products may be used, 36 gm. in 1000 c.c. distilled water.

M-72. Eosin Methylene Blue Agar, Levine.—

Dissolve 10 gm. Bacto-peptone
2 gm. dipotassium phosphate
10 gm. lactose
15 gm. agar
in 1000 c.c. distilled water.

Add in the order named 2 c.c. of 2% aqueous eosin Y solution. Mix well and add 0.5 c.c. of 2% aqueous solution of methylene blue. Mix well and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Pour in plates.

This formula is duplicated by BBL and Difco eosin methylene blue agar of Levine, 37.5 gm. dissolved in 1000 c.c. distilled water.

M-73. Brilliant Green Agar, Kristensen-Kauffmann.—

Meat or yeast extract	3 to 5 gm.
Peptone	10 gm.
Sodium chloride	5 gm.
Lactose	10 gm.
Sucrose	10 gm.
Phenol red	0.08 gm.
Brilliant green	0.0125 gm.
Agar	20 gm.
Distilled water	1000 c.c.

Sterilize in an autoclave. The final reaction should be about pH 7.2.
BBL and Difco duplicate this formula.
Dissolve 58 gm. in 1000 c.c. distilled water.

M-74. Aronson Medium.—

(a) Prepare extract nutrient agar (formula M-13) with 3% agar content, pH 7.4 to 7.6.
(b) Saturated alcoholic solution of basic fuchsin.
(c) 10% solution of sodium carbonate, anhydrous.
(d) 20% solution of sucrose.
(e) 20% solution of dextrin.
(f) 10% solution of sodium sulph*ite*, anhydrous.

Melt the agar (a) and add, to each 100 c.c., 5 c.c. of the carbonate solution (c).
Sterilize in flowing steam for 15 minutes.
While still hot, add, in order
5 c.c. sucrose solution (d)
5 c.c. dextrin solution (e)
0.4 c.c. fuchsin solution (b)
2 c.c. sodium sulphite solution (f)
Boil for a few minutes, then pour in plates. Store in dark and use within 3 days.

M-75. Bismuth-Sucrose Mannitol Agar, Wilson and Reilly.—

(a) **Reagent.**—

(1) Dissolve 100 gm. sodium sulph*ite* in 500 c.c. of boiling distilled water.

Dissolve 30 gm. bismuth ammonium citrate "scales" in 250 c.c. boiling distilled water.

Mix the two solutions and boil for 2 minutes. Let cool.

(2) Dissolve 50 gm. sucrose
and 5 gm. mannitol in 250 c.c. distilled water.

Dissolve 15 gm. sodium bicarbonate, anhydrous, in 400 c.c. distilled water. Mix the two solutions.

Add solution (2) to solution (1). Keep in a dark well-stoppered bottle.

(b) **Sodium Carbonate Solution.—**

Dissolve 53 gm. sodium carbonate, anhydrous,
in 400 c.c. distilled water.

(c) **Base.—**

Dissolve 10 gm. peptone
5 gm. sodium chloride
20 gm. agar
in 1000 c.c. distilled water.
Add 10 c.c. sodium carbonate solution (b)

Sterilize in the auotclave for 20 minutes at 15 pounds pressure. If necessary, adjust the reaction to pH 8.6.

(d) **Medium.—**

Melt 100 c.c. of the base (c), cool to 60° C., and
add 20 c.c. bismuth reagent (a)
2 c.c. 1:1000 phenol red solution
2 c.c. absolute alcohol.
Mix and pour in plates. Store in the dark. Use within 4 days.

M-76. Tetrathionate Broth, Mueller.—

(a) **Iodine Solution.—**

Dissolve 20 gm. of potassium iodide in 100 c.c. of distilled water by grinding in a mortar.

Add slowly and with constant grinding 25 gm. iodide. Keep in a dark glass-stoppered bottle.

(b) **Broth.—**

Peptone	10 gm.
Beef extract	3 gm.
Distilled water	1000 c.c.

Dissolve by heating. Adjust the reaction to pH 7.4.
Sterilize in the autoclave at 15 pounds pressure for 20 minutes.

(c) **Sodium Thiosulphate Solution.—**

Dissolve 50 gm. sodium thiosulphate
in 100 c.c. of distilled water by heating.

(d) **Pulverized Calcium Carbonate, U.S.P.—**

Method.—

Add the thiosulphate solution (c) to 900 c.c. of the broth (b).

Place in 10 c.c. quantities in test tubes.

Add to each tube about 0.5 gm. of calcium carbonate on the tip of a tongue depressor, and boil for 5 minutes.

Cool to room temperature and add 0.2 c.c. of the iodine solution (a) to each tube.

Do not sterilize.

BBL and Difco desiccated tetrathionate broth base may be used.

M-77. Tetrathionate Broth With Brilliant Green, Kauffmann.—

For each 1000 c.c. of the broth described in formula M-76 (b) add
10 c.c. of 1:1,000 aqueous solution of brilliant green "certified,"
and 25 gm. dried oxgall Difco.
Adjust the reaction again.
Prepare the medium in the same manner as that of Mueller, formula M-76.

M-78. Selenite F Medium, Leifson.—

Peptone	5 gm.
Lactose	4 gm.
Anhydrous sodium hydro-selenite	4 gm.
Combination of mono- and disodium phosphate	10 gm.
Distilled water	1000 c.c.

Adjust the reaction to pH 7.0 to 7.1.

Place 8 to 10 c.c. quantities in large test tubes, so that the column of the fluid is 5 to 7 cm. high.

Plug and sterilize by steam sterilization for 30 minutes.

Difficulties are frequently encountered in managing the correct proportions of phosphates and selenite. It is suggested, therefore, that the medium be obtained in its dehydrated form from the Baltimore Biological Laboratory, Baltimore, Md. The dehydrated powder is highly hygroscopic. Excess heating during sterilization may prove detrimental to the medium. If it is tubed and sterilized properly, it keeps well, either at refrigerator or room temperature. If the fluid turns red, or if a large red sediment occurs, decomposition of the medium has set in, and the medium has lost its usefulness.

M-79. Double Sugar Iron Agar, Kligler.—

To each 100 c.c. of melted extract nutrient agar (formula M-13), pH 7.4,
add 1 gm. lactose
0.05 gm. ferric ammonium citrate
0.05 gm. sodium thiosulphate
0.1 gm. dextrose
and 0.5 c.c. of a 0.5% watery solution of phenol red.

Tube and sterilize for 20 minutes at 15 pounds pressure in an autoclave.

Slant with long butts and short slants.

This medium may be purchased from Difco or Baltimore Biological Laboratory. Dissolve 52 gm. of the powder in 1000 c.c. distilled water.

M-80. Triple Sugar Iron Medium (T.S.I.), Hajna.—

Polypeptone	20 gm.
Lactose	10 gm.
Sucrose	10 gm.
Dextrose	1 gm.
Sodium chloride	5 gm.
Ferrous ammonium sulphate	0.2 gm.
Sodium thiosulphate	0.2 gm.
Agar	13 gm.
Phenol red	0.025 gm.

The medium may be purchased in dehydrated form from Baltimore Biological Laboratory or from Difco. Use 59.4 gm. per liter.

M-81. Braun Media, Modified.*—

(a) **Basic Solution.**—

Proteose-peptone No. 3 *or* Trypticase	20 gm.
Meat extract	1 gm.
Sodium chloride	2 gm.
Disodium phosphate, c.p.	2.254 gm.
Monopotassium phosphate, c.p.	0.09 gm.
Magnesium sulphate, c.p.	0.02 gm.

Dissolve in 1000 c.c. distilled water.

(b) **Sterile 3% solution of agar** in distilled water.

(c) Add to each 100 c.c. of basic solution (a)

Tryptophan	0.02 gm.
Cystine	0.004 gm.
Lactose	1 gm.
Sucrose	1 gm.
Sodium or ammonium lactate	1 c.c.

Adjust the reaction to pH 7.6.

Add 0.4% brom thymol blue ---------- 1 c.c.

(d) Mix equal parts of melted agar (b) and solution (c).

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*Schweiz. Ztschr. allg. Path. 8: 13, 1941.

(e) Tube, sterilize in autoclave at 15 pounds pressure for 15 minutes, and allow to solidify with long slants. This is **"Tube A."**

(f) Add to each 100 c.c. of basic solution (a)

Mannitol	2 gm.
Sodium thiosulphate, c.p.	0.1 gm.
Sodium or ammonium lactate	1 c.c.

Adjust the reaction to pH 7.6.

Add 0.4% brom thymol blue ---------- 1 c.c.

(g) Proceed as under (d) and (e), but allow the medium to solidify in a vertical position. This is **"Tube B."**

M-82. Motility Medium, Tittsler and Sandholzer.*—

Tryptose	5 gm.
Beef extract	3 gm.
Agar	5 gm.

Dissolve in 1000 c.c. distilled water.

Adjust the reaction to pH 7.4.

Tube and place in an autoclave at 15 pounds pressure for 15 minutes.

M-83. ''H''-Broth, Hajna and Damon.—**

Thiotone *or* Tryptone	10 gm.
Beef extract	3 gm.
Dextrose	1 gm.
Sodium chloride	5 gm.
Dipotassium phosphate	2.5 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.2 ±.

Suspend 21.5 gm. of the BBL or Difco powder in a liter of distilled water.

Mix thoroughly.

Warm slightly, if necessary to obtain solution.

Dispense in 5 or 6 c.c. amounts in 16 by 100 mm. test tubes, or in 3 c.c. amounts in 13 by 100 mm. tubes.

Sterilize by autoclaving at 121° C. (15 pounds pressure) for 15 minutes.

M-84. Semisolid Enteric Medium.†—

Thiotone	10 gm.
Beef extract	3 gm.
Sodium chloride	5 gm.
Agar	4 gm.
Gelatin	80 gm.
Sodium citrate	2 gm.
l-Cystine	0.2 gm.
Ferrous ammonium sulphate	0.2 gm.
Distilled water	1000 c.c.

The final reaction should be pH 6.9 ±.

Add 104.4 gm. of the dehydrated medium, BBL, to a liter of water preheated to about 50° C.

Heat over boiling water until the gelatin dissolves.

Boil for about 2 minutes.

Dispense the medium in 13 by 100 mm. test tubes. Regular 16 or 18 mm. test tubes may be used.

Sterilize in an autoclave at 118° C. for 15 minutes.

The medium should be inoculated by stabbing the center to one-third or one-half the depth of the medium.

It is recommended that cultures be incubated at 30° C. for 2 days or more.

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*J. Bact. 31: 575, 1936.

**Pub. Health Rep. 65: 116, 1950.

†Edwards and Bruner: U. Kentucky, Circ. 54: 1942; and Hajna: Pub. Health Lab. 8: 36, 1950.

M-85. Lactose Indicator Broth.—

Thiotone	10 gm.
Trypticase	10 gm.
Beef extract	3 gm.
Sodium chloride	5 gm.
Sodium citrate	2 gm.
l-Cystine	0.2 gm.
Lactose	20 gm.

Dissolve in 1000 c.c. distilled water.
Adjust the reaction to pH 7.4.

Add *either* 0.4% brom thymol blue	5 c.c.
or 0.4% brom cresol purple	5 c.c.

Distribute into Kolmer tubes, about 1 or 1.5 c.c. per tube.
Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

M-86. Triple Sugar Motility Medium, Felsenfeld and Young.—

Dissolve

Proteose-peptone No. 3 or Trypticase	10 gm.
Sodium chloride	3 gm.
Lactose	10 gm.
Sucrose	10 gm.
Salicin	5 gm.
Agar	3 to 3.5 gm.

in 1000 c.c. distilled water by boiling for a few minutes.
Adjust the reaction to pH 7.4 to 7.6.
Add 2 c.c. of 0.4% brom thymol blue
and 10 c.c. of Andrade indicator.
Tube with long butts and short slants.

Sterilize in the autoclave for 20 minutes at 10 to 12 pounds pressure. Test for sterility by incubating for 24 hours and observing for growth of bacteria.

Inoculate by deep stab.

M-87. Fulton Broth.*—

Dissolve 5 gm. adonitol
5 gm. aesculin
5 gm. salicin
5 gm. sucrose
0.05 gm. ferric ammonium citrate ''brown scales''
in 1000 c.c. of Difco-purple broth base.
Tube and sterilize in the autoclave for 15 minutes at 12 pounds pressure.

M-88. Semisolid Mannitol, Felsenfeld.—

Dissolve 10 gm. proteose-peptone No. 3 *or* trypticase
5 gm. sodium chloride
10 gm. mannitol
3 to 3.5 gm. agar
in 1000 c.c. distilled water by boiling.
Adjust the reaction to pH 7.4 to 7.6.
Add 4 c.c. of 0.4% brom cresol purple.
Tube with long butts.
Sterilize for 25 minutes at 15 pounds pressure in the autoclave.

In a recent modification, 0.05 gm. each of ferric ammonium citrate and sodium thiosulphate are added to each 100 c.c. of the medium.

M-89. Semisolid Mannitol With Hydrogen Sulphide Indicator.—

Thiotone	20 gm.
Yeast extract (*see* M-14)	3 gm.

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*Chilton and Fulton: J. Lab. & Clin. Med. **31**: 824, 1946.

Sodium chloride	5 gm.
Mannitol	10 gm.
Agar	4 to 5 gm.
Sodium thiosulphate	0.2 gm.
Ferric ammonium citrate	0.2 gm.

Dissolve in 1000 c.c. distilled water by heating.

Adjust the reaction to pH 7.4.

Add 0.4% brom thymol blue ---------- 5 c.c.

Tube in Kolmer tubes.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Inoculate by stabbing.

Take unusual care that the medium is not allowed to dry.

M-90. Urease Broth, Rustigian and Stuart.*—

Urea	20 gm.
Monopotassium phosphate	9.1 gm.
Disodium phosphate	9.5 gm.
Yeast extract	0.1 gm.
Phenol red	0.01 gm.

Use 3.87 gm. of the BBL or Difco powder per 100 c.c. of distilled water.

When the powder has dissolved, filter the medium through a sterile bacteriologic filter, either a Seitz or a candle filter.

Distribute the broth in 1 or 2 c.c. amounts in small sterile tubes.

M-91. Urease Broth With Lactose.—

Before sterilization by filtration, to each 100 c.c. of medium M-90 add 2 gm. of lactose.

M-92. Methyl-Red Voges-Proskauer Medium.—

Peptone *or* Polypeptone	5 gm.
Dipotassium phosphate, anhydrous	5 gm.
Dextrose	5 gm.
Distilled water	1000 c.c.

Dissolve the peptone and the phosphate by gentle heating. Adjust the reaction to pH 7.0 to 7.2.

Add the dextrose.

Distribute in 5 c.c. amounts into tubes.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Baltimore Biological Laboratory and Difco desiccated products marked "M.R.—V.P. Medium" are good substitutes for the above formula. Dissolve 17 gm. in 1000 c.c. distilled water.

M-93. Simmons Citrate Agar.—

Dissolve 1 gm. monoammonium phosphate
1 gm. dipotassium phosphate
0.2 gm. magnesium sulphate
2 gm. sodium citrate
5 gm. sodium chloride
in 1000 c.c. distilled water.
Add 20 gm. agar.

Soak for 15 minutes. Dissolve by heating, and adjust the reaction to pH 6.8.

Add 5 c.c. of 0.4% aqueous solution of brom thymol blue.

Tube and sterilize in the autoclave at 15 pounds pressure for 20 minutes.

Slant with long butts. Inoculate only the slants.

BBL and Difco desiccated powder, 24.2 gm. to 1000 c.c. distilled water, may be used.

M-94. Hippurate Broth.—

Dissolve 1 gm. sodium hippurate
in 100 c.c. beef infusion broth (formula M-4).

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*Proc. Soc. Exper. Biol. & Med. 47: 108, 1941.

Sterilize by filtration through a Seitz or Berkefeld filter.

Place, with sterile precautions, into sterile test tubes, 3 c.c. in each tube

M-95. Micromedium, Bertacchini, Modified.—

Dissolve 20 gm. peptone
3 gm. yeast extract
5 gm. sodium chloride
5 gm. agar
in 1000 c.c. distilled water.

Adjust the reaction to pH 7.2 to 7.4.

Add 10 c.c. Andrade indicator (page 1421)

and 2 c.c. of 0.4% brom thymol blue solution.

Distribute in 10 c.c. quantities in test tubes.

Sterilize for 20 minutes at 15 pounds pressure in the autoclave.

For use, melt and add to each tube 2 c.c. of 10% sterile solution of carbohydrate.

Mix well. Cool to 50° C., and add to each tube 0.5 c.c. of the culture to be tested.

Mix well.

Use a different sterile pipette for each tube. Place 0.3 c.c. quantities of each mixture into sterile Petri dishes. A number of different culture specimens may be put in the bottom of each dish provided that each 0.3 c.c. quantity is distinctly separated from the others, and each quantity is properly labeled.

Incubate at 37° C. and observe after 2, 4, 6 (and 8) hours for acid and gas formation, using the microscope.

M-96. Buffered Glycerol, Teague and Clurman.—

Sodium chloride, U.S.P.	4.5 gm.
Anhydrous dipotassium phosphate	3.1 gm.
Anhydrous monopotassium phosphate	1.0 gm.
Glycerol, U.S.P.	300.0 c.c.
Distilled water	700.0 c.c.

Dissolve the sodium chloride and the phosphates in the water, add the glycerol, adjust to pH 7.2 using phenol red as indicator.

Place in specimen bottles, filling the bottles less than one-half full with the fluid. Sterilize for 30 minutes at 15 pounds pressure.

M-97. Buffered Glycerol Water.—

Sodium chloride	4.5 gm.
Dipotassium phosphate, anhydrous	3.1 gm.
Monopotassium phosphate	1.0 gm.
p-Aminobenzoic acid	0.05 gm.
Cysteine hydrochloride	0.05 gm.
Glycerol	300.0 c.c.
Distilled water	700.0 c.c.

Dissolve the ingredients in the distilled water and adjust the reaction to pH 7.2.

Distribute and sterilize in the same manner as M-96.

M-98. Alkaline Sea-Salt Water, Venkatraman and Ramakrishnan.[*]—

(a) Dissolve 27 gm. sodium chloride
1 gm. potassium chloride
3 gm. magnesium chloride $(MgCl_2 \cdot 6H_2O)$
in 100 c.c. distilled water.

(b) Dissolve 12.405 gm. boric acid
14.912 gm. potassium chloride
in 800 c.c. hot distilled water, then dilute to 1000 c.c. with distilled water.

(c) 8% aqueous solution of sodium hydroxide.

For use, mix 20 c.c. sea salt solution (a)
250 c.c. boric buffer (b)
135.5 c.c. sodium hydroxide solution (c)

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[*] Indian J. M. Res. 29: 681, 1941.

in enough distilled water to make 1000 c.c.

Distribute in 10 c.c. quantities in screw-capped bottles of 30 c.c. capacity.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

M-99. Potato Dextrose Agar.—

Mix: Infusion from 200 gm. potatoes with
20 gm. dextrose and
15 gm. agar.

Suspend 39 gm. of BBL or Bacto potato dextrose agar in 1000 c.c. of cold distilled water.

Boil for 1 or 2 minutes to dissolve. Sterilize in the autoclave for 20 minutes at 15 pounds pressure. Final reaction is pH 5.6.

The label of each package of Bacto potato dextrose agar specifies the quantity of sterile tartaric acid (10% solution) that is to be added to each 100 c.c. of the sterile melted medium to adjust the reaction to pH 3.5.

Add the tartaric acid to the medium, mix well, and pour plates.

The medium should never be heated after the acid has been added, since heating in the acid state will hydrolyze the agar, and destroy its ability to solidify.

M-100. Malt Extract Agar.—

Dissolve 20 gm. agar in 800 c.c. distilled water by boiling.

Dissolve 25 gm. malt extract in 200 c.c. of distilled water.

Mix the two solutions. Distribute into bottles or tubes.

Sterilize in the autoclave for 30 minutes at 15 pounds pressure.

Difco malt agar, 45 gm. dissolved in 1000 c.c. distilled water, is the same as this medium.

M-101. Wort Agar.—

Maltose, technical	12.75 gm.
Malt extract, Difco	15.00 gm.
Dextrin, Difco	2.75 gm.
Glycerol	2.35 gm.
Dipotassium phosphate	1 gm.
Ammonium chloride	1 gm.
Bacto-peptone	0.78 gm.
Bacto-agar	15 gm.

Suspend 50 gm. of Bacto wort agar in 1000 c.c. cold distilled water.

Boil for a few minutes to dissolve.

Distribute into tubes or flasks as desired and sterilize in the autoclave for 15 minutes at 15 pounds pressure.

Final reaction is pH 4.8.

Due to the high acidity of the medium the heating process should be completed in as short a period of time as possible. Excessive heating of this acid medium causes a breaking down of the agar, resulting in inability to solidify properly when cool. Normally medium prepared from Bacto wort agar is soft and is ideal for planting purposes. However, if a medium of solidity satisfactory for streaking is desired, it can be prepared by using 60 gm. of Bacto wort agar per 1000 c.c. of water.

M-102. Sabouraud Agar I.—

Dissolve 20 gm. agar
in 1000 c.c. distilled water by heating.

While hot, add 10 gm. peptone and 40 gm. maltose.

Boil until dissolved. Adjust the reaction to pH 6.0 to 6.2.

Distribute into bottles or tubes.

Sterilize in the autoclave for 30 minutes at 15 pounds pressure.

Difco and BBL Sabouraud maltose agar, 65 gm. dissolved in 1000 c.c. distilled water, are reliable desiccated products.

M-103. Sabouraud Agar II.—

This differs from formula M-102 in that it contains dextrose instead of maltose.

M-104. Emerson Agar.[*]—

Beef extract	4 gm.
Gelysate	4 gm.
Sodium chloride	2.5 gm.
Yeast extract	1 gm.
Dextrose	10 gm.
Agar	20 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.0 ±.

Suspend 41.5 gm. of the BBL powder in a liter of distilled water.

Allow to stand for 5 minutes, and mix thoroughly.

Heat, with occasional agitation, and boil for 2 minutes to insure solution.

Dispense and sterilize at 118° to 121° C. for 15 minutes.

M-105. Littman Medium.[†]—

Oxgall	15 gm.
Gelysate and Polypeptone	10 gm.
Dextrose	10 gm.
Agar	16 gm.
Crystal violet	0.01 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.0 ±.

Suspend 51 gm. of the BBL powder in a liter of distilled water.

Allow to stand for 5 minutes.

Mix until a uniform suspension is obtained.

Heat with occasional agitation.

Boil for 2 minutes.

Dispense, and sterilize by autoclaving at 118° C. (12 pounds pressure) for 15 minutes.

Cool to 46° C.

Add 30 units of streptomycin per c.c. of medium (see *note*).

For best results, isolation plates should be made with about 30 c.c. of medium per plate. Plates should be allowed to stand, preferably for about 6 hours, before using.

NOTE: Littman medium should be used *without* streptomycin when the presence of *Nocardia asteroides* or any other organisms having sensitivity to streptomycin is suspected.

M-106. Mycophil Agar.—

Phytone	10 gm.
Dextrose	10 gm.
Agar	16 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.0 ±.

Suspend 36 gm. of the BBL dehydrated medium in a liter of distilled water. Allow to stand for 5 minutes and mix thoroughly.

When the suspension is uniform, heat with occasional agitation, and boil for about 2 minutes.

Dispense, and sterilize at 118° C. (12 pounds pressure) for 15 minutes.

M-107. Cornmeal Agar.—

Heat 40 gm. yellow cornmeal in 500 c.c. distilled water for 1 hour at 65° C.

Dissolve 15 gm. agar in 500 c.c. distilled water by boiling.

Mix the two solutions. Dispense into tubes or flasks.

Sterilize in the autoclave for 25 minutes at 15 pounds pressure.

BBL cornmeal agar without dextrose, 21 gm. dissolved in 1000 c.c. distilled water, may be used.

Difco cornmeal agar with dextrose, 19 gm. dissolved in 1000 c.c. distilled water, may be used instead of the above formula.

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[*] Emerson, Whiffen, Bohonos, and DeBoer: J. Bact. 52: 357, 1946.

[†] Science 106: 109, 1947.

M-108. Brilliant Green Lactose Broth.—

Dissolve 10 gm. peptone
10 gm. lactose
200 c.c. ox-bile
in enough distilled water to make 975 c.c.

Adjust the reaction to pH 7.2 to 7.4.

Add 13.3 c.c. of 0.1% solution of brilliant green, certified.

Place in 10 or 20 c.c. quantities in test tubes containing inverted fermentation vials.

Sterilize in the autoclave for 20 minutes at 12 pounds pressure.

A suitable dehydrated medium is made by Difco and BBL (brilliant green bile broth, 2%). Dissolve 40 gm. of this medium in 1000 c.c. distilled water and tube in 10 c.c. quantities when 1 c.c. or less of inoculum is to be used.

Dissolve 60 gm. in 1000 c.c. distilled water and tube in 20 c.c. quantities when the inoculum is to be 10 c.c.

M-109. Lauryl Sulphate Broth, Mallman and Darby.—

Dissolve 20 gm. tryptose *or* trypticase
5 gm. lactose
2.75 gm. dipotassium phosphate
2.75 gm. monopotassium phosphate
5.00 gm. sodium chloride
0.10 gm. sodium lauryl sulphate
in 1000 c.c. distilled water.

Adjust the reaction to pH 7.2.

Distribute in 10 to 20 c.c. quantities in test tubes. Place inverted fermentation vials in the tubes.

Sterilize for 15 minutes at 15 pounds pressure in the autoclave.

To use the dehydrated BBL or Difco product, lauryl sulphate tryptose broth, dissolve 35.6 gm. in 1000 c.c. distilled water, to test samples of 1 c.c. or less in 10 c.c. medium.

M-110. Dextrose Meat Extract Agar.—

To 100 c.c. of extract agar (formula M-13) with 1.5% agar, pH 7.2, add before sterilization 0.1 gm. dextrose.

Tube and sterilize as usual.

Difco proteose tryptone agar, and BBL dextrose extract agar, 34 gm. dissolved in 1,000 c.c. distilled water, may be used for this formula.

M-111. Dextrose Meat Extract Milk Agar.—

(a) Prepare extract agar with dextrose according to formula M-110.

(b) Dissolve 10 gm. Difco or BBL skim milk in 100 c.c. distilled water.

Before sterilizing agar (a), add to each 100 c.c. 1 c.c. of skim milk (b).

Tube and sterilize as usual.

M-112. S.F.* Medium, Hajna and Perry.—

Dissolve 20 gm. tryptone *or* trypticase
5 gm. sodium chloride
5 gm. dextrose
4 gm. dipotassium phosphate
1.5 gm. monopotassium phosphate
0.5 gm. sodium azide
8 c.c. of 0.4% brom cresol purple solution
in 1000 c.c. distilled water.

Place in 10 c.c. quantities in test tubes and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

The desiccated BBL and Difco products are used by dissolving 36 gm. in 1000 c.c. of distilled water.

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*Fecal streptococci.

M-113. Yeast Peptone Glucose Extract Agar, Jamieson.*—

To 100 c.c. of Difco tryptone glucose extract agar (formula M-110) add before sterilization

0.4 gm. yeast extract, Difco
1.0 gm. skimmed milk
1.0 gm. agar.

Heat to dissolve. Place in 4.5 c.c. quantities in flat 1-ounce bottles with screw caps.

Sterilize in the autoclave at 15 pounds pressure for 30 minutes.

Store in the refrigerator.

M-114. L Agar, Vera.[1]—

Lactalysate	20 gm.
Sodium chloride	2 gm.
Sodium citrate	3 gm.
Dextrose	9.5 gm.
Agar	13.5 gm.
Distilled water	1000 c.c.

The final reaction should be pH 7.0 ±.

Suspend 48 gm. of the BBL powder in a liter of distilled water.

Allow to stand for 5 minutes, and mix until a uniform suspension is obtained.

Heat gently with occasional agitation.

Boil for 1 or 2 minutes, or until solution is complete.

Dispense, and autoclave at 118° C. (12 pounds pressure) for 15 minutes. DO NOT OVERHEAT.

M-115. Stock Culture Agar, Difco.—

Beef heart infusion	500 gm.
Difco proteose peptone	10 gm.
Bacto gelatin	10 gm.
Bacto dextrose	0.5 gm.
Bacto isoelectric casein	5 gm.
Disodium phosphate	4 gm.
Sodium citrate	3 gm.
Bacto agar	7.5 gm.

Dissolve 50 gm. of the desiccated product in 1000 c.c. distilled water.

Tube and sterilize in the autoclave for 20 minutes at 15 pounds pressure.

Final reaction will be pH 7.5.

Cystine trypticase base, BBL (M-6), serves the same purpose.

M-116. Pleuropneumonia Medium, Norman and Kuhn.*—

(a) Trypticase	20 gm.
Agar	3.5 gm.
Sodium chloride	5 gm.
Sodium sulph*ite*	0.5 gm.
Cystine	0.5 gm.

Dissolve in 1000 c.c. distilled water by heating.

Add yeast extract	5 gm.

Dissolve by heating.

Adjust the reaction to pH 8.0.

Dispense into tubes, 4 c.c. per tube.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Before use, melt, and add to each tube 1 c.c. of sterile rabbit serum, which has been inactivated by heating at 52° to 56° C. for 30 minutes.

(b) Same as medium (a), but with 2% agar, is used for plates.

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*Canad. Pub. Health J. 37: 90, 1946.

[1] J. Bact. 54: 14, 1947.

[2] J. Bact. 58: 270, 1949.

M-117. Pleuropneumonia Media, Difco.—

PPLO Enrichment Broth, PPLO Agar, and Bacto-PPLO Serum Fraction are commercially available.

M-118. Dextrose Starch, Serum Agar, Brown and Nunemaker.*—

(a) Dissolve 65 gm. of Difco dextrose starch agar in 1000 c.c. distilled water by heating.

Adjust the reaction to pH 7.6.

Distribute in flasks in 100 c.c. amounts.

Sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Before use, melt and add 10 to 15 c.c. of serum or ascitic fluid to each flask.

(b) To prepare a solid medium, add 0.5 gm. of agar to each flask before sterilization.

M-119. Penicillin Assay Agar Base.—

Beef extract	1.5 gm.
Yeast extract	3 gm.
Peptone	6 gm.
Agar	15 gm.

Dissolve in 1000 c.c. distilled water by heating.

Adjust the reaction to pH 6.8; for streptomycin assay, to 8.0.

Distribute, and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Difco Penassay base agar and Mycin assay agar, and BBL base agar are superior to the homemade media.

M-120. Penicillin Assay Broth.—

Beef extract	1.5 gm.
Yeast extract	1.5 gm.
Peptone	5 gm.
Dextrose	1 gm.
Sodium chloride	3.5 gm.
Dipotassium phosphate	3.68 gm.
Monopotassium phosphate	1.32 gm.

Dissolve in 1000 c.c. distilled water by gentle heating.

Adjust the reaction to pH 7.2; for streptomycin assay, to 8.0.

Distribute and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Difco Penassay broth and Mycin assay broth, and BBL antibiotic assay broth are superior to the homemade media.

M-121. Penicillin Seed Agar.—

Beef extract	1.5 gm.
Yeast extract	3 gm.
Peptone	6 gm.
Pancreatic digest of casein	4 gm.
Dextrose	1 gm.
Agar	15 gm.

Dissolve in 1000 c.c. distilled water by heating.

Adjust the reaction to pH 6.8.

Distribute and sterilize in an autoclave at 15 pounds pressure for 15 minutes.

Difco Penassay seed agar and BBL seed agar are superior to the homemade media.

M-122. Yeast Beef Agar.—

This medium differs from medium M-121 in that it contains 0.1% dextrose. It is commercially available from Difco and BBL.

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*Bull. Johns Hopkins Hosp. 70: 201, 1942.

REAGENTS AND REACTIONS

Throughout this section, reagents and reactions are numbered as R-1, R-2, etc.

R-1. Motility

Motility of bacteria and other organisms may be determined by examining hanging drop preparations of broth cultures of the organisms. (See page 1467 for technic.)

Another very reliable method for testing for motility is to inoculate semisolid media by stabbing with a deep stab down to the bottom of the tube. Use formulas M-53, M-82, M-84, M-88, or any other semisolid medium containing 0.3% to 0.5% agar. Nonmotile organisms grow only along the stab line. Motile organisms spread out into the medium.

R-2. Decomposition of Fermentable Substances

Decomposition of fermentable substances (carbohydrates, alcohols, glucosides, etc.) is usually indicated by change in color of the indicator present in the culture medium. Such media as M-5, M-6, M-7, M-84, M-95, etc., contain indicators which are added during preparation. Clostridia are tested in media to which the indicators are often added after incubation, since they might reduce them.

One may observe for the formation of acid or of acid and gas. Acid formation in liquid media is indicated by a change in the color of the medium. Acid formation in solid media is indicated by a change in the color of that portion of the medium immediately surrounding the bacterial colonies. At times the acid formation is profuse and the entire plate or tube assumes the acid color.

Gas formation may be observed in liquid media, in semisolid media, or in solid media. In semisolid media, gas formation is indicated by formation of bubbles. This method of testing is preferred.

To determine gas formation in liquid media, place an inverted vial in each tube of the medium before sterilization. During sterilization this inverted tube fills up with medium. Gas formation during fermentation collects in the sealed end (top) of the inverted tube. The quantity of gas can be measured if desired.

In solid media gas formation is detected by the formation of bubbles in the media. In some cases gas formation is so profuse that the entire tube of medium is blown up, sometimes out of the tube.

It is important to remember that gas cannot be produced without acid formation. The color of the indicator, therefore, is always changed to the acid color when gas bubbles are present. If gas bubbles are observed without color change, either the vial, in the liquid medium, is too large and did not fill up during sterilization, or the solid medium broke for some other reason, such as drying. At all events, such bubbles are the result of some technical error and are not due to gas formation by the bacteria.

For practical purposes, it is enough to test gas formation in dextrose only. If an organism, especially Enterobacteriacea, forms gas from this carbohydrate, it may be considered a gas-former. This permits simplification of laboratory procedures, especially when fermentation vials are employed instead of the more convenient semisolid media which show gas formation by the development of bubbles. Vials are difficult to clean and often get lost during the cleaning procedures. Should vials be used, they must be put in dextrose only; the other carbohydrate media are tubed without them. An exception is, naturally, work with water and milk.

R-3. Hemolysis

It is often necessary to test the ability of bacteria to hemolyze blood. To test aerobic organisms, streak blood agar plates formula M-28 or M-29. To test anaerobic organisms, streak yeast blood agar formula M-14. Incubate the plates for 24 and 48 hours.

Hemolysis occurs as a clear (dissolved) zone surrounding the bacterial colonies. For a detailed description of types of hemolysis, refer to pages 1487 ff.

Poured plates give the most reliable results. Add the bacterial suspension or liquid culture to the blood agar while it is still liquid (temperature must be as low as possible) and pour plates. Allow to solidify and incubate for 24 and 48 hours, then observe for hemolysis.

If a quantitative estimation of hemolysis is desired, use the tube method. Mix equal parts of bacterial suspensions or broth cultures with suspensions of red blood cells. Incubate at 37° C. in a water bath.

The concentration and source of red blood cells used in the test will vary with different organisms. The routine procedure is to mix 0.5 c.c. of the culture with 0.5 c.c. of 5% rabbit red blood cells and incubate for 4 hours.

One must never test for hemolysis in media containing carbohydrates.

R-4. Coagulase Formation

Preparation of the Culture.—

(a) Use a 24-hour broth culture of the staphylococci to be tested; *or*

(b) Transfer the typical opaque, round, white or golden orange colony from the original plate where the isolation was made to an agar slant, and incubate for 12 hours, or slightly longer.

Test for Coagulase.—If the broth culture is used, add 0.5 c.c. of the 24-hour culture to 0.5 c.c. of citrated human blood plasma.*

If the agar slant is used, transfer one loopful of the growth to 0.5 to 1 c.c. of plasma in a small (75 by 10 mm.) tube.

Place in a water bath at 37° C. and observe frequently over a period of three hours, observing for coagulation. If the 37° C. water bath is not available a hot air incubator is satisfactory, but the test must be carried out for a six-hour period. A second reading is made in six to eight hours in the water bath, and in twelve to eighteen hours in the incubator.

Formation of a clot indicates a positive test. The size of the clot is not important. It is desirable that the clot form in three hours. Antibiotic treatment may result in loss of coagulase. Levine uses two different concentrations of plasma—undiluted whole plasma and plasma, one volume, diluted with two volumes of saline. Bacto-coagulase plasma solution is a reliable product.

R-5. Fibrinolysin Test

Fibrinolysin is an active substance, produced by some bacteria, notably streptococci pathogenic for man, which dissolves human fibrin.

Inoculate a nutrient agar plate (M-15) containing 10% plasma, heated for 10 minutes at 56° C.

Incubate for 48 hours at 37° C. and observe for zones of clearing surrounding the colonies. This indicates formation of fibrinolysis.

R-6. Reaction in Milk

Inoculate Ulrich milk M-11 or anaerobic iron milk M-51.

Record the formation of acid, gas, clotting, and peptonization.

R-7. Liquefaction of Gelatin

The classic procedure was to inoculate gelatin, incubate at 18° to 20° C. and observe for liquefaction.

A satisfactory method is to inoculate gelatin formula M-84 in screw-capped tubes and incubate at 37° C. After 48 hours, one week, two weeks, and three weeks, put the tubes in the refrigerator for 30 minutes. If the gelatin does not solidify it has been decomposed.

Another method is to streak the organisms on Chapman-Stone agar, formula M-46, and incubate for 24 hours.

Observe for cleared zones around the colonies which indicate liquefaction of the gelatin.

R-8. Reduction of Nitrates

Sulphanilic Acid-Naphthylamine Reaction.—

Reagents.—

1. Nitrate broth, formula M-8, and anaerobic nitrate broth, M-50.
2. Dissolve 0.8 gm. of sulphanilic acid in
   100 c.c. of 5 N acetic acid, sp. gr. 1.041.

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*Dehydrated plasma for the coagulase test is now available from Warner-Chilcott, 9 East 62nd St., New York 21, N. Y., and from Difco Laboratories, Detroit, Mich.

3. Dissolve 0.5 gm. alpha-naphthylamine
in 100 c.c. of 5 N acetic acid, sp. gr. 1.041.

Technic.—

Inoculate tubes of nitrate broth and incubate for 3 days.

To the growth add 0.1 c.c. of the sulphanilic acid solution (2).

Then add, drop by drop, alpha-naphthylamine solution (3) until a red or maroon color appears. This is a positive reaction.

R-9. Hydrogen Sulphide Formation

Hydrogen sulphide formation is detected by black discoloration of a proper indicator, such as, lead acetate, ferric citrate, bismuth citrate, etc. These substances are added to the medium (M-51, M-52, M-56, M-79, M-80, M-84, etc.).

Some workers prefer to use *lead acetate papers.—*

Dissolve 10 gm. lead acetate, neutral, in 50 c.c. boiling distilled water.

Immerse strips of filter paper, about 2½ by ¼ inches, in this solution.

Dry in the incubator and store in well-stoppered bottles.

Inoculate broth cultures or slants containing a peptone favorable to the production of hydrogen sulphide, such as thiopeptone or proteose-peptone No. 3.

Incubate and then insert a strip of the lead paper between the plug and the neck so that it reaches into the tube but does not touch the medium.

Observe the paper for 48 hours for evidences of blackening or browning which indicate H_2S formation.

R-10. Bile Solubility Test

Inoculate neopeptone extract broth M-2 or trypticase soy broth M-3, or M-6, with the organisms to be tested.

Preparation of Bile for Use in the Bile Solubility Test.—

The bile may be obtained from the abattoir. It is collected in a sterile bottle.

Filter through sterile filter paper.

Place in 5 c.c. quantities in sterile ampules.

Seal in the flame.

Sterilize in the autoclave at 15 pounds pressure for thirty minutes.

If a precipitate forms after a time, it can be removed by centrifuging the bile, and decanting the supernatant fluid.

Filter about 5 c.c. of the original culture through sterile filter paper, or make a suspension of one of the colonies in 5 c.c. sterile saline.

Add 0.5 c.c. of sterile bile. Incubate for 10 minutes to one-half hour at 37° C.

Examine by means of the hanging drop.

Pneumococci dissolve.

Sodium Taurocholate or Sodium Desoxycholate Solutions for the Bile Solubility Test.—Solutions of pure bile salts are preferable to ox bile because they may be sterilized and the concentration better controlled. Note that heat-killed pneumococci are not bile-soluble. Sodium taurocholate or sodium desoxycholate is used in 10% solution for the bile solubility test.

Leifson Technic.[1]—Make up a 10% sodium desoxycholate solution with 1:50,000 Merthiolate as preservative. Add 2 drops of this solution to 1 c.c. of a 24-hour broth culture or saline suspension of the test organism. Make up a control of 1 c.c. of the culture and 2 drops of sterile saline solution. If the organism is a pneumococcus, the solution clears or the organisms are dissolved at once. Examine, however, for at least 10 minutes before calling the test negative.

Lauryl Sulphate Test, Bayliss.[2]—

To 0.9 c.c. of the culture add 0.1 c.c. of 2% sodium lauryl sulphate.

Incubate for 30 minutes at 37° C.

Pneumococci will dissolve in this time.

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[1] J. A. M. A. **104**: 213, 1935.

[2] J. Lab. & Clin. Med. 28: 748, 1943.

R-11. Hippurate Test

Inoculate hippurate medium formula M-94 and incubate for 24 hours.

Add an equal amount of the reagent:

2 c.c. concentrated hydrochloric acid
98 c.c. distilled water
12 gm. ferric chloride ($FeCl_3 \cdot 6H_2O$)

Observe for a 15-minute period for precipitation.

R-12. Indol Formation

Inoculate broth or semifluid agar containing no carbohydrates, but a peptone favorable for indol production, such as, tryptone or trypticase formula M-1 or M-50.

Incubate 16 to 24 hours (or longer) and test by one of the following methods:

(a) Kovacs.—

Dissolve 5 gm. *p*-dimethyl amino benzaldehyde
in 75 c.c. of amyl *or* butyl alcohol in a water bath at 55 to 60° C. When solution is complete
add 25 c.c. of concentrated hydrochloric acid.

Add 0.5 to 1.0 c.c. of this reagent to the culture and shake well.

A red ring forms within 5 minutes if the reaction is positive.

(b) Jacobs and Pincus.[1]**—**

Reagent.—

Dissolve 10 gm. trichloroacetic acid
in 30 c.c. chloroform.
Add 2 gm. *p*-dimethyl amino benzaldehyde.
Transfer to a separatory funnel.
Saturate by shaking with concentrated hydrochloric acid.

Technic.—

Shake the culture with 1/5 its volume of chloroform.
Separate the chloroform layer.
Add 1 drop of the reagent to each c.c. of this layer.
Keep at 50° C. for 30 seconds.
A pink or rose color indicates indol formation.

(c) Goré.—

Prepare test tubes with absorbent cotton plugs, and inoculate these culture tubes with the organism to be tested.

Moisten the plugs with a few drops of saturated solution of potassium persulphate.

Moisten the plugs with Ehrlich reagent (method b).

Push the plugs down to about 1 inch above the surface of the medium.

Place tubes in boiling water bath for 15 minutes.

A red color on the plugs indicates the presence of indol.

(d) Gnezda.—

Saturate a strip of filter paper with oxalic acid by dipping it into a warm saturated aqueous solution of oxalic acid.

Inoculate the culture medium, and when replacing the plug, insert the strip of paper between the plug and the neck of the tube.

Incubate the culture and observe after 24 and 48 hours for pink discoloration of the paper, which represents indol formation.

R-13. Methyl Red Test

Inoculate tubes of methyl red Voges-Proskauer medium formula M-92 and incubate for 3 days.

To each 5 c.c. of the medium add 2 drops of freshly prepared 0.04% solution of methyl red.

A yellow color indicates a positive reaction.

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[1] Science 102: 204, 1945.

R-14. Voges-Proskauer Test

Inoculate tubes of methyl red Voges-Proskauer medium formula M-92 and incubate for at least 48 hours. Then carry out one of the methods below.—

(a) Leifson.—

Reagent.—

Dissolve 1 gm. copper sulphate in 40 c.c. ammonia water
and add 10% sodium hydroxide, enough to make 1000 c.c. solution.

Technic.—

Add an equal volume of the reagent to the medium.
Let stand for 30 minutes without the plug.
If the reaction is positive an eosin color will develop.

(b) Barritt.—

Reagents.—

1. 5 % alcoholic solution of alpha-naphthol
2. 40% solution of potassium hydroxide

Technic.—

Transfer 0.6 c.c. of alpha-naphthol (1) to a test tube.
Add 1.0 c.c. of culture in methyl red Voges-Proskauer medium.
Add 0.2 c.c. of potassium hydroxide (2).

Shake. Observe for a 4-hour period. A pink or red color indicates a positive reaction.

(c) O'Meara.—

To the growth in the culture medium, add that amount of creatine which can be picked up on a knife point.
Add 5 c.c. 40% sodium hydroxide.
Shake for 2 to 5 minutes.
A pink color indicates a positive reaction.

(d) Rapid Method, Coblentz.[1]—

Reagents.—

1. Methyl red Voges-Proskauer medium formula M-92 tubed in 2 c.c. quantities.
2. 5% solution of alpha-naphthol in 95% ethyl alcohol.
3. Dissolve 40 gm. potassium hydroxide
   in 100 c.c. distilled water.
   Add 0.3 gm. creatine.
   Dissolve by shaking.

Technic.—

Make a heavy inoculation in the culture medium (1) from an agar slant and incubate at 30° C. for 6 hours.

Add 0.6 c.c. of alpha-naphthol solution (2) to each tube.

Add 0.2 c.c. of alkaline creatine (3).

Shake vigorously for 1 minute. Observe after 10 minutes for a red or pink color, which indicates a positive reaction.

R-15. Urease Test

(A) **Method of Rustigian and Stuart.[2]—**

Inoculate urease test medium formula M-90 and incubate not longer than 24 hours. A red color indicates urea decomposition.

(B) **Rapid Method of Anderson.[3]—**

Make a heavy inoculation of urease test medium formula M-90 tubed in 0.1 c.c. quantities by transferring the inoculum from a slant culture.

Incubate for 30 minutes and observe for pink to red color, which indicates urea decomposition.

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[1] Am. J. Pub. Health 33: 815, 1943.
[2] Proc. Soc. Exper. Biol. & Med. 47: 108, 1941.
[3] Science **101**: 470. 1945.

R-16. Cholera Red Test

Grow organisms in tryptone nitrate broth formula M-8 for 24 hours.

Add 0.3 c.c. concentrated sulphuric acid.

A red color indicates a positive reaction.

R-17. Citrate Utilization

Inoculate Simmon citrate agar formula M-93 by streaking the surface.

Incubate and observe for 3 days. The indicator turns blue when citrate is utilized.

R-18. Oxidase Test

Culture organisms on blood plates containing dextrose (M-28, M-29).

Reagent.—

Dissolve 0.1 gm. dimethyl-para-phenylenediamine hydrochloride or oxalate
in 10 c.c. distilled water.

Preserve in a glass stoppered bottle. Use within one week.

Technic.—

Flood the plate with about 2 c.c. of the reagent.

Oxidase-positive organisms, such as, *N. gonorrhoeae, N. intracellularis,* many bacilli, etc., give a lavender color, which turns purple, and, finally black.

Tetramethyl-para-phenylenediamine hydrochloride is less toxic for Neisseriae but more expensive. Dimethyl-para-phenylenediamine oxalate is more stable.

R-19. Acrolein Reaction

Schiff Reagent.—

Dissolve 0.125 gm. basic fuchsin
in 100 c.c. distilled water by gentle warming.
Cool to room temperature and decant.
Add to the fluid 50 c.c. freshly prepared 4% sodium sulphite
and 4 c.c. concentrated hydrochloric acid
and enough water to make a total volume of 250 c.c.
After 5 minutes at room temperature, filter rapidly through filter paper.
Store in a dark bottle at room temperature.

This reagent will keep for one month.

Technic.—

Grow organisms in thioglycollate glycerol broth M-55.

Transfer 0.5 c.c. of this growth to 2.5 c.c. thioglycollate broth in a Kolmer tube.

Incubate at 37° C. until decolorized, but not longer than 24 hours.

Add to a separate tube 0.9 c.c. of this culture and 0.1 c.c. of fresh Schiff reagent.

A magenta color within 15 minutes at room temperature denotes a positive reaction.

R-20. Lecithinase Reaction

Grow organisms anaerobically on Nagler's plate or its modification M-57.

Observe after 48 and 60 hours for precipitation around the colonies.

CULTURE METHODS

With the exception of body fluids, material collected from cases or carriers is rarely inoculated directly into tubed fluid media for diagnostic purposes. Stools, pus, and swabs (except when investigating diphtheria, tuberculosis, and protozoan infections) are plated on media in Petri dishes. In many cases, however, an enrichment fluid, such as tetrathionate broth, is inoculated first; then, after sufficient incubation, plates are streaked from this fluid. In other cases, a preservative, such as that of Hirschberg for *N. gonorrhoeae* or buffered glycerol saline for bacillary incitants of diarrhea, is used, chiefly for the transportation of the specimens. Thus, the bacteriologic investigation becomes a planned procedure. Best results are attained when a proper scientific selection of methods is combined with reliable, sterile technic, and rapid work.

Preserving Fluids must be inoculated with a proper amount of material. Too much material impairs the efficacy of the preservative, while too little may cause false negative results. The technic of the use of preservatives for transportation of material is described in detail in the section dealing with diagnostic methods.

Enrichment Fluids must be handled in a manner similar to that used for preserving fluids. In addition to the proper amount of inoculum, temperature and length of incubation are of paramount importance in order to prevent the overgrowth of "contaminating" microorganisms.

Plating Media must be prepared and kept under conditions prescribed for the respective medium. Plating is the only reliable technic for isolation of bacteria in pure culture. It is usually done by streaking the material on the surface of media in Petri dishes.

POINTS IN IDENTIFYING BACTERIA

Make a survey examination in a hanging drop preparation to determine morphology and grouping. Then make a Gram stain to study staining characteristics.

Transplant to various culture media, preferably in Petri dishes to study colonies. When various characteristics of the bacteria are learned, use other culture media for further study, or transfer to sugar-containing broths or special media for fermentation reactions.

Note characteristic pigments, odors, effect on blood, effect on blood serum, egg, whether gelatin is liquefied. Observe whether growths are slight, moderate, or abundant; transparent or opaque; adherent; whether they emulsify easily or with difficulty, whether colonies are confluent or discrete.

If necessary, inoculate animals, make bile solubility tests, or agglutination tests.

Always try to place an organism in its genus, then proceed to identify the species.

Streaking Plates

Only proper streaking can insure isolated colonies. It is a sheer waste of time and material to simply smear a specimen over a plate or a section thereof, expecting to make a bacteriologic diagnosis. The art of streaking a plate is not inborn in bacteriologists, but has to be learned by hard daily practice.

If the streaks "did not come out" so that no isolated colonies were obtained, a fresh specimen must be requested. An honest laboratory worker will avoid issuing a negative report when distinctly isolated colonies have not been seen on the plates.

Re-plating may help in an emergency but should not be practiced.

Method I. "Streak" Method

Have on hand a Petri dish containing the culture medium on which the organism to be isolated grows characteristically.

Pick up a loopful of material to be planted, and streak out over the upper part of the culture medium across from one side to the other. Be careful to touch the surface of the medium, but do not dig into the agar.

Flame the platinum wire.

Streak out at right angles to the original line of inoculation making the streaks about ½ inch distant from each other.

Flame the platinum wire.

Turn the plate, and streak at right angles to the parallel lines just made, so that the result is a series of lines forming small squares over the surface of the medium.

Flame the platinum wire.

Throughout the procedure, use precautions to eliminate contamination with air bacteria.

Turn the plate **upside down** and place in the incubator for twenty-four hours or longer at 37° C., until a good growth has been obtained.

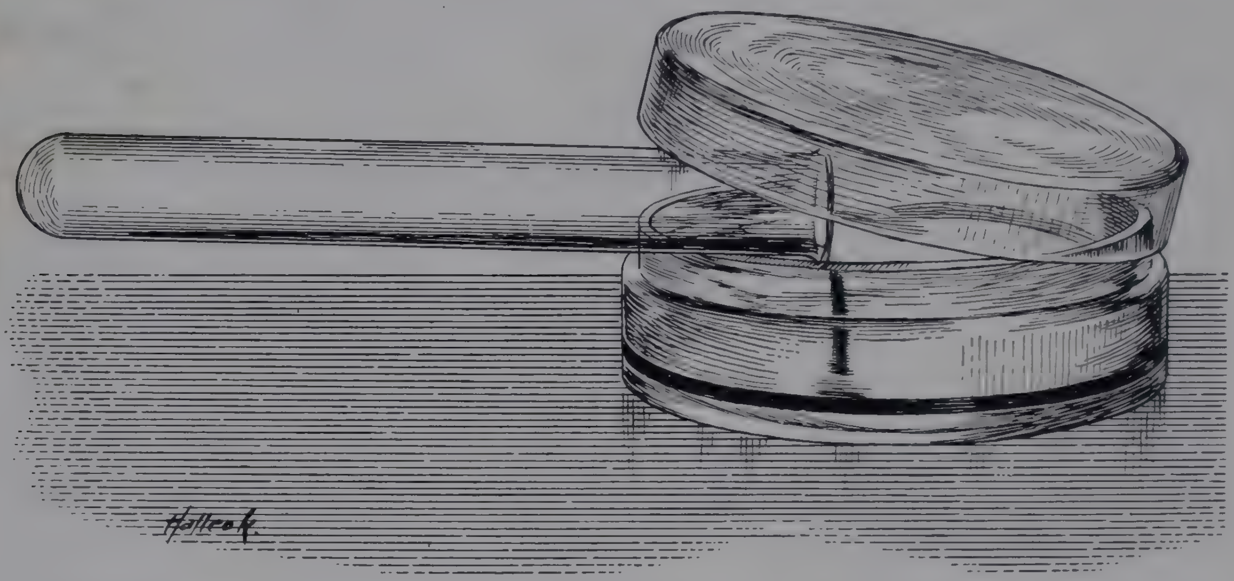

Fig. 336.—Pouring melted agar into a Petri dish.

By fishing out a typical colony under the microscope, and inoculating on a slant or another Petri dish of the same medium as that used in the isolation process, a pure culture is obtained.

The amount of material taken in the original loopful must be gauged by the possible number of bacteria in the specimen. There should be few enough bacteria so that at the ends of the streaks distinct colonies appear.

If there is a growth of bacteria in the centers of the squares formed by the streaking process, these, obviously, are contaminants.

If the medium is very moist, the water of condensation will carry the bacteria over the surface of the plate, and render it unfit for isolation purposes. Care should be taken, therefore, to eliminate as much of the water as possible.

Method II. "Clock Streak"

Pick up a loopful of the material to be plated and streak out over the upper ⅕ of the plate.

Flame the loop and cool it by stabbing it once or twice into the medium in some place which was not streaked.

Turn the plate about 70 degrees.

Take some of the material from the streaked area by drawing the loop through it in a straight line, and streak out over the next ⅕ of the plate. Return the loop to the streaked portion, draw a line through it, and streak the material out.

Flame the loop and cool it by stabbing it once or twice into the medium in some place which was not streaked.

Turn the plate about 70 degrees.

Take some material from the part last streaked and streak out as before.

Repeat this procedure once more. See Fig. 337.

Proceed in the manner described in **Method I.**

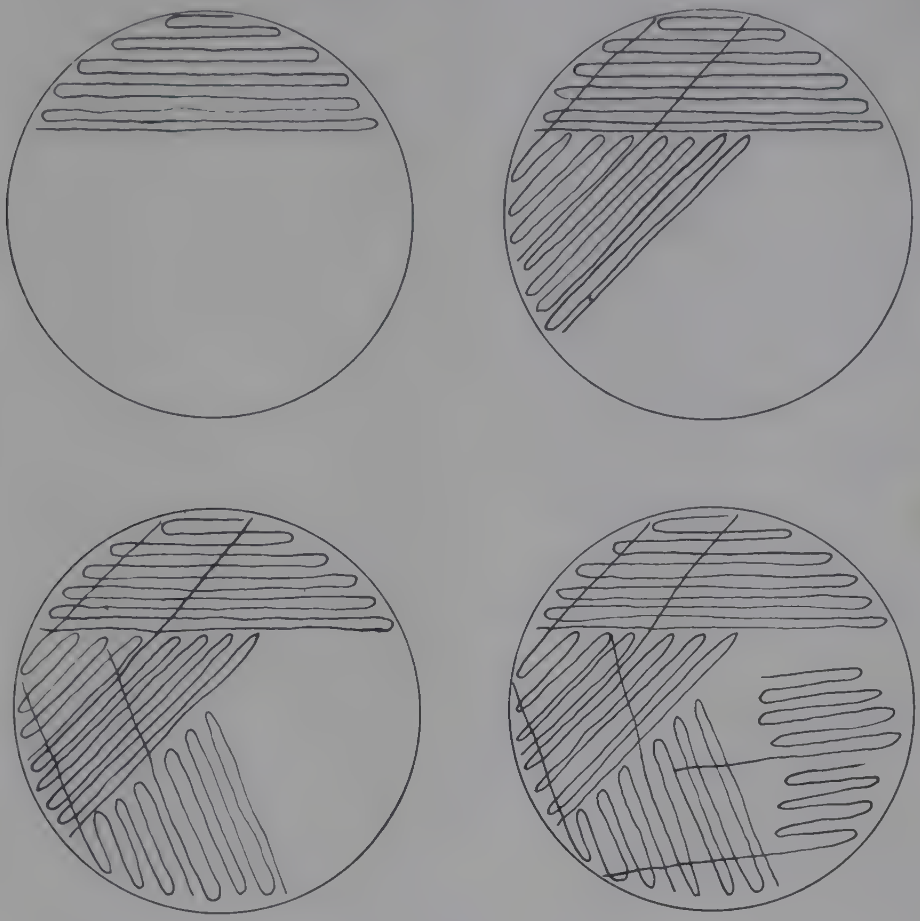

Fig. 337.—Steps used in performing the clock streak. (Courtesy Dr. Oscar Felsenfeld.)

Method III. "Z" Method

Prepare a Petri dish of the desired culture medium, and allow it to solidify.

Pick up a loopful of the material to be planted, and streak over the surface of the agar in such way that a letter "Z" is formed and covers the surface of the plate. Flame the loop.

Streak out from the initial line of inoculation; flame the loop; streak out from another point on the line; flame the loop; continue this process until many streaks have been made, flaming the loop between each streak. Flame the loop.

Turn the plate upside down in the incubator at 37° C. for twenty-four hours or longer, and proceed in the manner described under **Method I.**

Method IV. "Compass" Method

Prepare a Petri dish of the desired medium, and allow it to solidify.

Along the radius of the circle (Petri dish) place three small loopfuls of the material to be isolated.

Prepare a piece of capillary glass by heating glass tubing in the flame, pulling it out to a capillary, then, while still hot, bending it to a 45 degree angle. (These capillaries may be prepared, wrapped, and sterilized, ready for use.)

Using the capillary as a compass, describe a circle over the surface of the medium in such way that the three loopfuls of material are spread in a thin layer over the entire surface.

Burn the capillary in the flame.

Turn the plate upside down in the incubator at 37° C. for twenty-four hours or longer, then proceed in the manner described in **Method I.**

Method V. Spreading With a Glass Rod

Prepare glass rods by bending them at a 90 degree angle. The short end of the rod should be about ⅓ of the diameter of the plates used, the longer arm about 5 inches long.

Wrap each rod in paper and sterilize in the dry heat sterilizer.

Place a drop of the material to be examined on the plate of culture medium.

Rub it in with the sterile glass rod.

Without taking fresh material, rub the same glass rod over the surface of a second plate.

Proceed as directed in **Method I.**

Method VI. ''Spreading'' Method

This method is useful when the material to be isolated is in a liquid.

Pour a plate of the desired medium, and allow to solidify.

Place 0.05 c.c. (1 drop) of the material to be isolated on the surface of the agar. A sterilized 1 c.c. pipette graduated in 0.01 c.c. may be used for measuring the drop.

Allow the drop of material to spread over the surface of the agar in such way that it entirely covers the medium.

Turn the plate upside down, and incubate at 37° C. for twenty-four hours or longer.

Proceed in the manner described under **Method I.**

This method can be used only if few bacteria are present.

Some Precautions in Plating

The surface of the plate must be dry but not dried-out.

To avoid contamination, do not keep the plates open too long. Lay the lid on the laboratory table upside down. Hold the bottom of the Petri dish (the part which contains the medium) in a vertical position, with the medium turned toward the worker, to prevent settling of organisms from the air.

When plates are examined or inoculated, it is imperative that the worker does not speak. Contaminated fishings are sometimes due to the selection of colonies which are too near the others. Contamination during the ''fishing'' process is often due to conversation while the covers are removed. Cultures are exposed during this time, and precautions must be taken not to contaminate them.

Do not cut the medium with the inoculating loop. If this should occur on soft media, interrupt the streak and take fresh material from another part of the surface of the plate for continuance of the streak.

Always keep Petri dishes upside down except when streaking them or during inspection or ''fishing.''

''Fishing'' or ''Picking'' Colonies

For most purposes, the colony to be transferred from the plate to a differentiating medium is selected macroscopically. Use daylight coming in through the window, and not direct sunshine. Using a wax pencil, draw a circle on the bottom of the Petri dish around the colony to be fished.

Flame a platinum or nichrome wire.

Raise the bottom of the Petri dish to eye level, holding it in a vertical position.

Cool the needle by touching it once or twice to the medium in an area which was not streaked and on which there is no growth. Or, better, allow it to cool in the air.

Pick up the colony taking care not to touch another colony in so doing. If using selective media which retard the growth of contaminants but do not kill them, pick up only the center of the colony.

Replace the Petri dish in its cover.

Holding the needle between the index finger and the thumb, and using the same hand, pull out the plug of the tube containing the differentiating medium held in the other hand. To accomplish this, use the little finger of the hand, bending it against the palm, with the top of the cotton plug between the finger and the palm of the hand.

Flame the neck of the tube.

Inoculate the medium in the tube.

Again flame the neck of the tube and replace the plug.

Flame the needle.

If the hands of the worker are unsteady, it is best to rest the elbows on the table while picking colonies. It is also possible to lean the hand holding the plate against the hand holding the needle by touching the proximal parts of both antithenars.

When "fishing" with the aid of a microscope is indicated, the following procedure is recommended.

Focus the colony on the Petri dish with lid open using the 16 mm. objective of the microscope. Leave the Abbé condenser in the microscope. Keep the edge of the colony in sharp focus.

Flame a platinum or nichrome loop.

Steady the arm on the table.

Holding the platinum loop between the first and second fingers, to make it easy to manipulate, and guiding it with the thumb, place the hand on the stage of the microscope with the little finger resting on the stage. Place the wire over the area of the colony. Look into the microscope at the colony and introduce the wire until it appears in the field of vision.

Remember that an invert image is presented, and that a loop introduced from the right side appears on the left side of the microscopic field.

Pick up the colony that has been selected.

Withdraw the wire, taking care that it does not touch any spot on the medium.

With a little practice this can be accomplished with a great deal of ease.

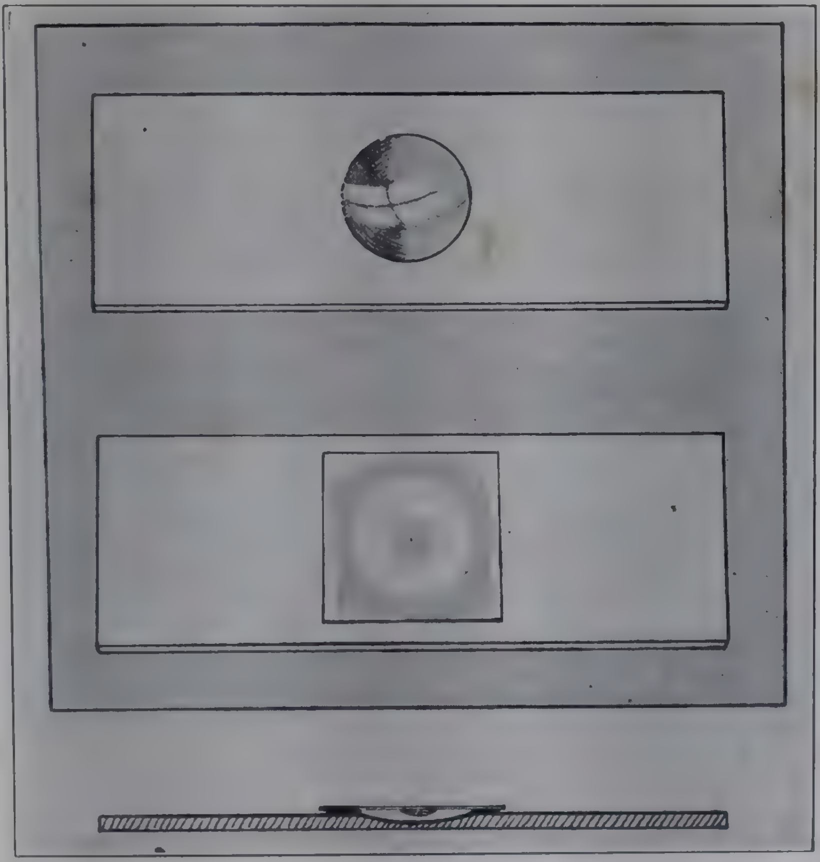

Fig. 338.—Preparing a hanging-drop specimen.

Making and Examining Hanging-Drop Preparations

The hanging-drop slide should be fat-free, and free of lint. Clean a ⅞ inch cover glass, taking care not to touch the glass with the fingers.

Place a small quantity of petrolatum around the edge of the cover glass.

Pick up a loopful of material to be examined, and place it in the center of the cover glass. Take care not to take too large a loopful, as it will touch the hanging-drop slide, and give a false appearance when examined.

Pick up the cover glass with the hanging-drop slide in such way that the loopful of material is in the center of the concavity of the slide.

Place the preparation on the microscope stage, and move it until the drop is directly under the 16 mm. objective.

Remove the Abbé condenser, use the plane surface of the mirror, shut off most of the light by manipulating the mirror, and focus up, until the edge of the drop is distinctly in focus.

Move the slide until the edge of the drop is in the center of the microscopic field.

Change to the 4 mm. objective and the concave surface of the mirror, lower the objective until it touches the slide, adjust the light, then focus up very slowly until the drop comes into view. By focusing for the edge of the drop, the worker has a definite line for which to look. Bacteria are too pale to afford a good medium for focusing.

Take care not to turn the fine adjustment of the microscope too far, as the objective might crash into the cover glass of the preparation and break it. There is no way of sterilizing the objective without injury, and since the preparation contains living bacteria, there is danger of contamination.

Inoculation of Culture Media in Slants in Test Tubes

When the stopper is removed from the first tube, it should be held between the third and fourth fingers, and care should be taken that the end of the stopper which is to go back into the tube does not touch anything.

The tubes should be held in a slanting position, and the mouths heated by holding them in the flame of a Bunsen burner for a short time.

The tube from which the specimen is taken must be held in a slanting position, and the mouth flamed.

A platinum or nichrome wire in a glass rod is heated red hot and cooled.

A portion of the material to be planted is picked up with this wire and is inserted into the second test tube.

Beginning at the bottom of the slant, inoculate in a zigzag motion over the surface of the slant, taking care to touch the medium, but not to dig into it.

Flame the wire to red heat after withdrawing it from the tube.

Flame the cotton stopper, flame the top of the test tube, then replace the stopper.

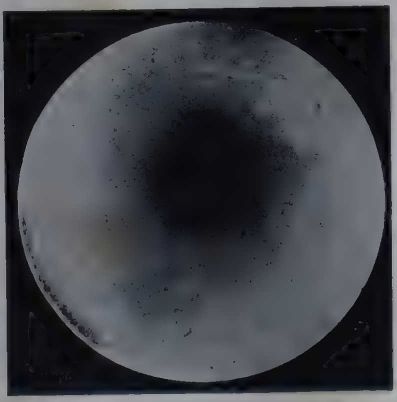

Fig. 339.

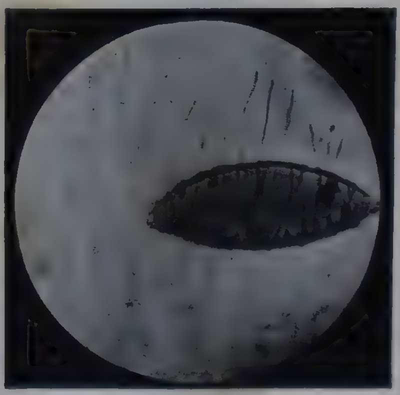

Fig. 340.

Fig. 339.—Colony of yeast from the air.
Fig. 340.—Contamination of cultures with molds and deep colonies.

Inoculation of Liquid Media

Using all sterile precautions (that is, flaming the mouths of the containers from which the specimen is taken, and the tube containing the liquid medium, and holding the plugs between the fingers), pick up a loopful of material to be planted, using a sterile platinum or nichrome wire. Make an emulsion of the material on the side of the tube just above the liquid. Replace the cotton plug, flame the wire, and mix the emulsion with the liquid by rotating.

Inoculation of Butts

Using all sterile precautions, that is, flaming the mouth of the tube from which the material is taken, and of the tube containing the medium, and holding the plugs between the fingers, pick up some of the material, using a sterile platinum or nichrome wire.

Introduce this material with one single stab into the medium, stabbing down to the bottom of the test tube. Replace the cotton plug and flame the wire.

Inoculation of Slants and Butts

This method is often used in examination of enteric organisms.

With the usual precautions, first stab the needle to the bottom of the medium, then streak the rest of the material on the needle over the surface of the slant.

When inoculating butts, take special care that the entire wire is well flamed (sterilized) and that contaminants from the holder are not added to the medium. This often occurs when using fluid media if the loop is shaken in order to force more material into the liquid.

Success in growing pure cultures depends greatly on proper plugging of the containers. If the medium is allowed to flow down the neck of tubes during the tubing process, particles of cotton often adhere to the glass. This greatly increases the chances of contamination.

SPECIAL CULTURE METHODS

Many organisms grow well under aerobic conditions. Some require anaerobic cultivation. It must be kept in mind that most aerobic organisms grow well also under anaerobic conditions. Thus not every bacterium which grows on anaerobic plates is an obligatory aerobe.

Making Anaerobic Cultures

1. Brewer Sodium Thioglycollate Medium

(Using a liquid medium without special apparatus) (Brewer Medium)

Brewer[1] has made use of several well-established principles and has developed a clear liquid medium which will remain anaerobic for more than one month when used in regular culture tubes without any type of seal. This medium possesses the unusual property of bringing about a reversible reaction; that is, if the medium is aerated by stirring or shaking, it again assumes an anaerobic state upon standing. It is called thioglycollate medium and is now available in powdered form from the Baltimore Biological Laboratory, Baltimore, Md. It is a facultative medium for the cultivation of obligatory anaerobes, microaerophiles, and aerobes (M-47, M-49).

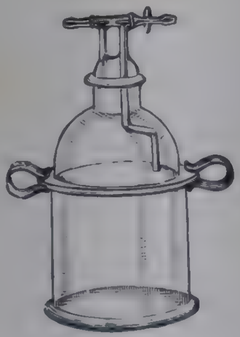

Fig. 341.—Novy anaerobic culture jar. (Courtesy E. H. Sargent & Co.)

Polypeptone broth containing 0.07 per cent agar serves as a base for this medium. It has been shown that small numbers of organisms grow much more readily if fractional percentages of agar are used, and Brewer stated that even this small percentage of agar lessens convection currents, thereby prolonging the anaerobiosis obtained without noticeably affecting the fluidity of the medium. The proper oxidation reduction potential is obtained by using 0.1% sodium thioglycollate. Methylene blue is added to serve as an indicator of the oxidation reduction potential. This small dye concentration (1:500,000) is nontoxic and exhibits no bacteriostatic action against any of the large group of organisms tested.

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[1] Brewer, John H.: J. Bact. 39: 10, 1940.

The medium has the usual infusion broth color except at the surface where an aerobic layer has a green color due to the methylene blue. In inoculating and handling, the medium may assume a green color throughout, but will change again, in a few minutes, to its usual amber color when the anaerobiosis is re-established. The medium needs only the careful handling given the usual aerobic media. The tubes may be rotated after inoculation to distribute the inoculum. The tubed medium makes possible a liquid medium, the surface of which is aerobic while the subsurface is anaerobic; hence, the medium possesses a variable degree of anaerobiosis, making possible the cultivation of aerobes, micro-aerophiles, as well as anaerobes, in a single medium. It well may be called a facultative medium. Brewer's media are described as M-47, M-49.

A Petri Dish Cover and Technic for Use in the Cultivation of Anaerobes and Micro-Aerophiles. (Brewer Method[1])

John H. Brewer, of Baltimore, Maryland, who originated the thioglycollate medium for the cultivation of anaerobes,* has originated a very satisfactory method for the surface cultivation of anaerobes and micro-aerophiles without the use of anaerobe jars, petrolatum seals, or chemicals other than those included in the medium itself. In our laboratories, we have followed this method with signal success. We have found it a very satisfactory method for cultivating *Clostridium tetani* and *Cl. perfringens.* We have had occasion to use this method in connection with an investigation of samples of ham which were responsible for a case of food poisoning. We found that the predominant organism in this case was *Staphylococcus aureus* and that this method showed an anaerobic development of this organism. It is to be recommended very highly.

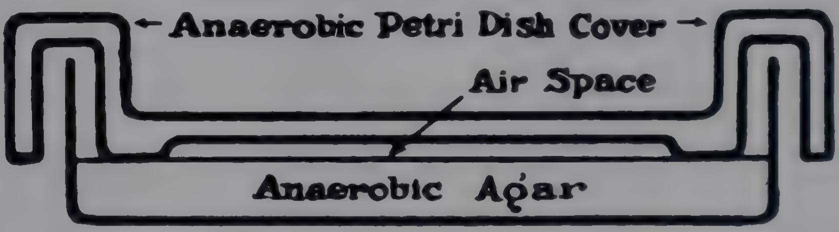

Fig. 342.—Cross section showing Brewer anaerobic Petri dish cover in use.

Technic.—

Pour any good infusion agar containing a satisfactory reducing agent into the usual Petri dish and allow to harden. Either a pour or streak plate may be made. After the agar has solidified, replace the Petri dish cover with the anaerobic lid which is so designed that it touches the agar at the periphery and results in trapping a small amount of air over the surface of the agar. The reducing agent in the medium uses up the oxygen in this small amount of air and an aerobic condition exists. The glass rim on the lid forms a seal with the moist solidified agar and no other seal is necessary. If 1 c.c. of 1:500 methylene blue is added to each liter of agar to act as an indicator, the reduced center of the medium in the dish becomes colorless, while the oxygenated periphery for about 5 mm. remains blue. For the preparation of Brewer's agar, see formula M-47 or M-49.

Brewer found that *Cl. tetani, Cl. novyi, Cl. septicum,* and *Cl. perfringens* give good surface colonies in 48 to 72 hours and that the plates may be incubated several days longer without drying out. In most cases the growth was much better than that obtained with the same culture in an anaerobe jar. If an unglazed porcelain top is used in pouring the plates, better isolation of surface colonies will be obtained. To facilitate opening the dish, the cover should be turned slightly to break the agar seal.

This technic may be used with the usual agar for obtaining partially anaerobic conditions for the cultivation of microaerophiles.

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*Lab. Digest, Feb., 1942.

[1] Brewer, J. H.: Science 95: 587, 2475, 1942.

2. Using the Novy Jar

The jar should be tested for air leaks before use. All parts with a possibility of leakage should be thoroughly covered with petrolatum.

Place the cultures in the jar, and clamp the lid in place. There should be a rubber pad between the upper and lower parts of the jar at the point of contact.

Turn the stopcock located at the top of the jar, to open it. Attach the end of rubber tubing to one end of the stopcock, and attach the other end of the tube to the gas outlet. Allow the gas to flow into the jar until all air has been expelled. Close the stopcock. Turn off the gas. The exhaust may be burned off if desired.

Place the jar in the incubator for three days or longer.

3. Using Gradwohl-Drucker Anaerobe Culture Jar

The apparatus consists of a standard quart Mason jar and a cast aluminum top with two stopcocks. This cast aluminum top seals upon the Mason jar. Rubber tubing connects the shorter stopcock to the gas jet. Place the inoculated tubes or Petri plates in the jar and replace the lid. Open both stopcocks. Fill the jar with illuminating gas from the gas jet, burning off the excess. Burn for 15 to 20 minutes, then turn off the fire stopcock, then the gas stopcock, then the gas jet. Place the jar in the incubator for 24 hours or longer until the desired growth of cultures is obtained.

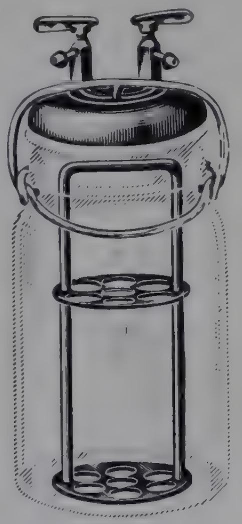

Fig. 343.—Gradwohl-Drucker anaerobic jar. (Courtesy Phillips-Drucker Co., St. Louis, Mo.)

4. Wright Method

Into a 100 c.c. lipless cylinder place about 1 inch of pyrogallic acid crystals.

Add enough 10% sodium hydroxide to dissolve the pyrogallic acid.

Place the culture tube in the dissolved pyrogallic acid. Immediately close the mouth of the cylinder with a rubber stopper. Place petrolatum around the stopper to make it airtight.

Place in the incubator for three days or longer.

5. Method of Consumption of Oxygen by a Second Bacterium

Inoculate half the surface of a Petri dish with the organism to be studied.

Inoculate the remainder of the plate with the *Bacillus subtilis* or *Serratia marcescens.*

Close the air space between the cover and the bottom of the Petri dish with modeling clay.

Place upside down in the incubator.

The *Bacillus subtilis* or *Serratia marcescens,* in growing, will consume the small supply of oxygen left in the plate.

The other organism, in this way, is afforded anaerobic conditions.

6. Sealed Tubes

Use tubes of any suitable medium, as, for example, semifluid agar formula M-50, chopped meat (M-12), or similar medium. Heat for a few minutes to expel the oxygen.

Cool and inoculate the medium.

Pour over the medium a layer of sterile liquid paraffin (petrolatum), using sterile precautions. The layer should be about ⅔ inch high.

Replace the plug and incubate.

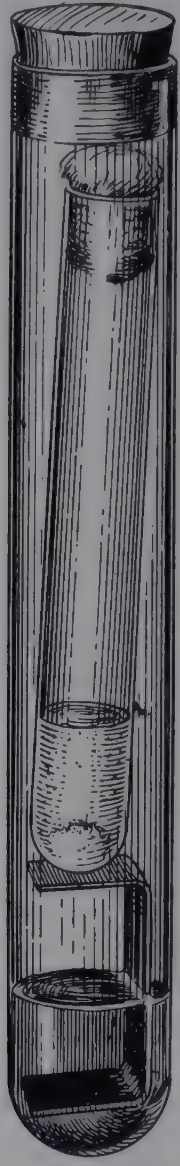

Fig. 344.—Wright method of making anaerobic cultures.

7. Spray Dishes

The Spray dish is a container, the lower part of which contains a ridge which divides the bottom of the dish. The upper section is similar to an extra heavy Petri dish and is used for preparing the culture. Pour the medium into the cover using sterile precautions.

Streak the medium with the inoculum.

Fill one half of the bottom of the Spray dish with 4 c.c. of a 20% solution of pyrogallic acid. Fill the other half with 10 c.c. of a 20% sodium hydroxide solution. Take great care not to mix the chemicals before closing the cover.

Place the lid, with the culture medium down, over the Spray dish so that the culture medium forms the lid. Fill the space between the cover and the Spray dish with modeling clay.

Shake the dish gently so that the pyrogallic acid and the sodium hydroxide mix, but take care that none of this solution splashes onto the culture medium. Incubate in the usual manner.

Cultivation in Carbon-Dioxide Atmosphere

Fig. 341 shows a type of apparatus which is used to admit carbon dioxide under strong pressure into a culture jar. Such apparatus is necessary for the cultivation of microorganisms which require carbon dioxide under high tension as, for example, *Brucella abortus*.

Other microorganisms require carbon dioxide under less tension (Neisseriae). There are several methods for producing such an atmosphere.

(1) Place inoculated plates in a container which can be tightly covered; for example, desiccator jar, Pyrex preparation jar, Petri dish container, coffee can, or any other available container. Place a lighted, smokeless candle in the container and close the lid. The flame consumes the oxygen and enough CO_2 is produced for the culture needs of the bacteria.

(2) Place a handful of oats in a one-quart container (e.g., desiccator) and moisten the oats with water. Fasten the lid of the container and incubate. This method may cause contamination of the culture plates with molds.

(3) Place in one-half of the bottom of the Spray dish 0.5 gm. sodium bicarbonate with enough distilled water to dissolve it. In the other half place 3% sulphuric acid. Place the lid containing the inoculated culture medium over the Spray dish, medium side down, and seal with modeling clay. Shake the dish from side to side to mix the contents in the bottom of the dish, taking care not to splash the liquid on the medium.

Egg Culture Methods

The Use of Chick Embryos in the Diagnosis of Certain Infections[1]

Egg culture methods are used not only for the propagation and isolation of viruses, but also for diagnosis of many bacterial diseases. The special value of this living medium has been demonstrated for culturing tubercle bacilli and such delicate organisms as *Hemophilus influenzae, H. pertussis, Brucella abortus, Pasteurella tularensis,* meningococci, and pneumococci.

Due to the speed of growth of bacteria in this medium, the method is an important factor in the early diagnosis of various bacterial diseases. In any laboratory which is set up for this work, there must be someone on duty 24 hours a day, to begin the procedure as soon as the specimen is delivered, and thereby permit proper examination, which hinges on the study of fresh materials, early diagnosis, and the subsequent establishment of proper treatment.

The egg as a culture medium has been applied particularly to the culture of spinal fluids in meningitis. The routine methods employed in many hospital bacteriologic laboratories have failed frequently to yield the etiological agent of this disease. The use of chick embryos is of special value when only a few organisms are present, or where the ordinary culture media might fail to detect them.

Most commercial chicken feed is now enriched with antibiotics; thus the source of the egg supply must be thoroughly checked. Antibiotics in the feed will diminish or prevent bacterial growth. Cortisone-treated chick embryos have been employed, and a marked increase in tissue reaction and stimulation has been noted. These cortisone-injected embryos seem useful for the cultivation of body fluids from persons under antibiotics or chemotherapy.[2]

Use a saline suspension of Cortone acetate made by emulsifying commercial cortisone. Drop 0.1 or 0.2 c.c. of this commercial cortisone from a tuberculin syringe onto the chorioallantoic membrane at least 2 hours before inoculating with the test suspension, which sensitizes the egg tissue for bacterial invasion. Not less than 2 eggs should be used per specimen.

Contaminated material may be freed from undesired bacteria by mixing 500 units of penicillin with or without 500 units of streptomycin with the sample, and allowing it to stand for 1 to 2 hours.

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[1] Wolf, Herma: Lab. Digest **15**: 6, 1952.

[2] Brueck and Buddingh: Proc. Soc. Exper. Biol. & Med. **80**: 589, 1952.

Injection of methylene blue, 1% aqueous solution, is an excellent aid for visualizing parts of the egg, especially during training.

The following methods are satisfactory for bacteriologic studies, using the chorioallantoic membrane technic.

(A) Material.—

(1) A reliable source of fertile eggs with 7- to 12-day-old embryos.

Some workers prefer to purchase eggs containing younger embryos and then keep them in a 39° C. incubator, with sufficient moisture, until they are ready for use.

(2) A candling device. This may be purchased, or it may be homemade.

(3) A poultry incubator, 39 to 39.5° C., with egg trays and water container. A cardboard with holes in it may be used as an egg tray. A routine bacteriologic incubator will give good service.

(4) An apparatus to open the egg shell: a dentist's drill, a hand steel trocar, or a supply of safety razors fitted into a handle.

(5) A holder for egg inoculation: it may be combined with the candling device or it may be a special apparatus.

(6) Sterile tuberculin syringes with 27 gauge needles, or sterile capillary pipettes for inoculation.

(7) Sterile small forceps, preparation needles, and knife.

(8) Rubber suction bulb.

(9) Sterile cover glasses.

(10) Sterile paraffin-wax mixture.—

Paraffin	40 gm.
Beeswax	10 gm.
Castor oil	10 gm.

(11) Sterile saline, preferably containing 0.044% calcium chloride.

(B) Technics.—

(1) Burnet.[1]**—**

Incubate eggs in the poultry incubator, turning them over each day.

Candle 10- to 14- (preferably 12) day-old eggs to be sure that the embryos are alive. In the living egg, the embryos and the yolk sacs appear as dark bodies; the embryos are not too mobile when the eggs are turned, and the blood vessels are visible.

Mark the position of the embryo with a wax pencil on the eggshell.

Drill the eggshell over the natural air sac.

Drill a small window, about 2 sq. cm., in the side of the egg, where the chorio-allantoic vessels are visible.

Cover drillings with paraffin-wax mixture.

Put a drop of sterile saline on the fibrous membrane.

After a few minutes gently separate the chorio-allantois from the shell membrane.

With the rubber bulb, apply suction through the opening over the natural air sac, until the chorio-allantois falls off the shell membrane.

Completely open the window on the side of the egg, where now an artificial air sac has formed (after the chorio-allantois falls).

Using a tuberculin syringe or a capillary pipette, drop the inoculum on the chorio-allantois.

Seal well with paraffin-wax. Seal the opening over the former (natural) air sac.

Incubate the eggs for 6 days, observing them every day.

Using sterile technic, make smears from the surface of the chorio-allantois.

If histologic examination is desired, flood the chorio-allantois with the fixative, and remove after a few minutes.

(2) Hirst, Modified by Twyble and Mason.[2]**—**

Locate the embryo by candling.

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[1] M. Research Council, Spec. Ser. No. 220, 1936; J. Bact. 44: 241, 1942.

[2] J. Immunol. 49: 73, 1944.

Sterilize the egg shell with alcohol.

Make a small hole over the natural air sac on the blunt end of the egg, using a probe.

Make a second hole over the region where the chorio-allantoic vessels are visible.

Deposit the material (usually 0.1 c.c.), using a sterile tuberculin syringe, into the second hole just beneath the egg shell.

Seal the holes with Scotch tape and incubate as above.

(3) Goodpasture and Budding, Modified by Gallaher and Kurachi.[3]—

Do not sterilize the surface of the egg.

Drill only one hole, over the area of the chorio-allantoic vessels.

Remove the shell and the fibrous membrane with a sharp knife.

Deposit the material on the chorio-allantoic membrane with a syringe or a capillary pipette.

Ring the window with sterile paraffin-wax, then cover it with a flamed cover glass, and proceed as above.

(4) Allantoic Sac Inoculation.—

The technic of Beaudette and associates[4] is recommended.

Deposit the inoculum about 1 cm. beneath the inner shell membrane with a 22 gauge needle, entering under the air cell of the egg.

(5) Yolk Sac Inoculation.—

Beaudette and associates recommend that a small drop of thin oil be deposited on the inner shell membrane to clarify it. This method is effective after a cap of the shell over the air cell is removed.

Beginners will do well to use a methylene blue solution to learn how to penetrate into the different parts of the egg. Control with a strong light will aid in the inoculation.

In working with developing chick embryos, sterility is most important, since many bacteria will cause death of the embryo. Contamination occurs very easily. It is of paramount importance that the eggs come from a hatchery which has chickens free from pullorum and other diseases. The age of the eggs is also important. Younger eggs are better for yolk sac inoculation.

(C) Special Methods.—

(1) Isolation of Mycobacterium Tuberculosis by Inoculation of the Yolk Sac of Embryonated Eggs.—

Material from patients with suspected tuberculosis, as spinal fluid, pleural exudate, gastric washing, sputum, tissue, or urine, may be inoculated. Within 4 to 5 days, acid-fast bacilli are readily demonstrable even from specimens in which the microorganisms were not found by direct smear and in concentrated specimen.

Embryos after 5 to 8 days' preliminary incubation at 37° C. are suitable. The yolk sac is inoculated through a small drill hole in the shell (see above) by means of a syringe with a 20 or 22 gauge needle. After injection, the drill hole is closed with melted paraffin.

Spinal fluid requires no preliminary preparation. It must be collected under aseptic conditions. An inoculum of 0.2 c.c. is used per yolk sac. Inoculate 5 to 6 eggs.

For **pericardial and pleural exudates**, a bacteriologic sterility test must be made by inoculating blood agar plates (M-28, M-29) and thioglycollate broth (M-47, M-48) with 0.2 to 0.5 c.c. amounts of the test fluid. Simultaneously inoculate 0.2 c.c. of material per yolk sac. Bacterial contaminants are controlled by the addition of penicillin or bacitracin, 500 units of penicillin and 500 units of bacitracin per c.c.

Gastric washings and sputum must be specially prepared.

Digest these with equal parts of 4% sodium hydroxide at 37° C. until all mucus and visible particles are dissolved.

Centrifuge at 2000 to 3000 r.p.m. for 30 to 60 minutes.

Remove and discard the supernatant fluid.

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[3] Am. J. M. Technol. **10**: 2, 1944.

[4] Am. J. Vet. Res. **12**: 267, 1952.

Add a few drops of sterile 0.04% brom thymol blue.

Add to the sediment 6% sulphuric acid until green.

Suspend the sediment in 1 or 2 c.c. of saline containing 1000 units of penicillin per c.c.

Let stand for 2 hours in an incubator at 37° C.

Use 0.1 to 0.2 c.c. of this suspension per yolk sac as inoculum, and use 2 eggs routinely, or 3 if scanty growth is expected.

Tissues and exudates must be ground with sterile alundum and suspended in saline containing 1000 units of penicillin per c.c.; grind the tissue in a sterile mortar, using about 0.5 gm. of tissue and 3 to 5 c.c. of the saline. If the material is too thick for aspiration, dilute in saline containing 1000 units of penicillin per c.c. Inoculate at least 0.1 or 0.2 c.c. per each yolk sac, after letting it stand in an incubator at 37° C. for 2 hours.

Incubate inoculated eggs at 37° C.; turn them twice a day and observe at least once a day.

Examination.—

Inoculated eggs must be candled daily and discarded if the embryos die during the first 4 days. Beginning on the fourth day after inoculation, one embryo from each group should be examined daily.

Transfer the yolk sac, with aseptic precautions, to a sterile Petri dish.

Transfer approximately 1 c.c. of the yolk to a 2.5 by 10 cm. test tube.

Add 20 c.c. of 1% phenol.

Seal with a rubber stopper and shake vigorously for 5 minutes.

Allow to stand at least 2 hours.

Using a capillary pipette, carefully remove a small amount of the fatty layer at the surface, and smear it extremely thinly on the surface of one or more clean slides.

Allow to dry.

Fix in the flame.

Stain by the Ziehl-Neelsen method.

Examine microscopically for the presence of acid-fast bacilli.

In most instances, preparations made in this manner will contain typical acid-fast bacilli on the fourth day following inoculations.

To confirm the presence of *Mycobacterium tuberculosis,* inoculate guinea pigs, into the groin and intraperitoneally, with 0.5 to 1 c.c. from at least one yolk sac from a group of embryos used for the test. Tuberculin-test the guinea pigs at the end of three weeks, and sacrifice those with positive reactions. Proceed in the usual manner to examine for acid-fast bacilli in their organs.

(2) Meningitis.—

In many cases of meningitic infections, growth of bacteria can be demonstrated by egg inoculation when simultaneous agar cultures fail to permit their detection. Whereas growth on routine culture media is often sparse when few organisms are present in the specimen, and colonies do not develop for as long as 2 or 3 days, the presence of meningococci in the egg can be demonstrated within 10 to 24 hours after inoculation. This is a decided advantage for an early diagnosis.

Elimination of Gram-Negative Organisms

Method of Owen[1]

The presence of gram-negative organisms may interfere in the examination of wound secretions, pus, etc. Spreading Protei and Pseudomonades may impair the isolation of gram-positive cocci. Several methods have been devised for preventing the spreading of gram-negative bacilli. Use of potassium tellurite, as in medium M-41, or of sodium azide as in media M-21 and M-22, is good. Phenylalcohol plates (M-23) are helpful, too. The following method can be used if none of these other media are available.

(a) Culture pus or exudate in formula M-4.

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[1] J. Bact. 52: 353, 1946.

(b) After 18 to 24 hours, transfer 0.1 c.c. of the growth in tube (a) to two tubes of ground beef medium M-12 consisting of a layer, about 1 inch high, of ground meat and 15 c.c. of beef heart infusion broth M-4, one tube containing 0.1 c.c. of 10% acetic acid, the other 0.2 c.c. of 10% acetic acid.

(c) Incubate 18 to 24 hours.

(d) Streak on blood agar plates M-28 or M-29, containing 5 to 6% of 95% alcohol.

Standardization of Bacterial Suspensions (McFarland)

Make a set of nephelometric tubes by mixing 1% sulphuric acid, c.p., with 1% barium chloride, c.p., according to Table 143.

TABLE 143

TUBE	1% SULPHURIC ACID C.C.	1% BARIUM CHLORIDE C.C.	CORRESPONDING DENSITY OF BACTERIA APPROXIMATELY MILLION PER C.C.
1	9.9	0.1	300
2	9.8	0.2	600
3	9.7	0.3	900
4	9.6	0.4	1,200
5	9.5	0.5	1,500
6	9.4	0.6	1,800
7	9.3	0.7	2,100
8	9.2	0.8	2,400
9	9.1	0.9	2,700
10	9.0	1.0	3,000

The tubes must be of uniform size and of resistant glass.

Plug tubes with rubber stoppers and carefully seal with paraffin. Set the tubes up in a rack.

For estimation, compare bacterial suspensions with the standards.

This set serves to determine approximately the number of bacteria present in a saline suspension. To estimate the bacterial density in broth cultures, make the set by dissolving the sulphuric acid and the barium chloride in sterile broth.

Some more modern procedures use photelometers. Standardize the curve by using the McFarland standards, above. Select the filter according to the photelometer and the color of the fluid in which the organisms are suspended.

PREPARATION OF VACCINES

Vaccines are killed suspensions of bacteria in phenol-saline, Chloretone, etc. An autogenous vaccine is made from bacteria derived from a patient's lesion. A stock vaccine is made from stock cultures of bacteria kept in the laboratory.

Preparation of Materials Needed.—

1. Several ½ ounce colorless wide-mouth bottles, with corks, sterilized in the dry heat oven at 170° C. for 2 hours.

2. Phenol and saline solution, made as follows:

Distilled water	1,000.0	c.c.
Sodium chloride, c.p.	8.5	grams
Mix thoroughly until dissolved		
Melted carbolic acid	5.0	c.c.
or		
Liquid carbolic acid	5.74	c.c.

Mix thoroughly until the carbolic acid is dissolved.

Filter through filter paper into wide-mouth bottles.

Fill about ⅔ full. Plug with nonabsorbent cotton. Tie paper over the top.

Sterilize in the autoclave at 15 to 20 pounds pressure for thirty minutes.

3. Tubes of culture medium for the bacterial growth, or Kolle or Blake flasks.
4. Platinum or nichrome wire.
5. Sterile 1 c.c. ampules.
6. Sterile syringe for transferring finished vaccine to ampules.

Making the Cultures.—

Select the medium on which the bacteria present in the lesion produce the best growth. Inoculate two culture tubes or one Kolle or Blake flask with material from the lesion. Record on record sheet.

Incubate at 37° C. for twenty-four to forty-eight hours, or until a good growth is obtained.

Identify the bacteria present by smears and colony study.

Record on the record sheet the type or types of bacteria present.

Proceed to isolate pure cultures by plating.

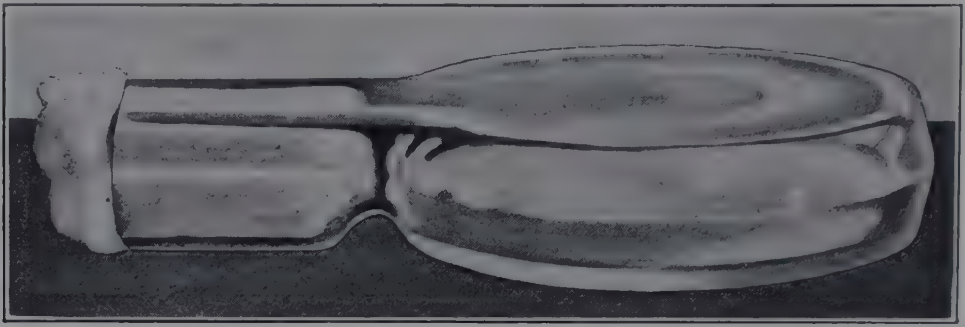

Fig. 345.—Kolle flask for vaccines.

Preparing the Suspension.—

Using a sterile dropper, or a sterile pipette, transfer a few c.c. of sterile phenol and saline solution to the surface of the two slants of bacteria. Enough phenol-saline solution should be used to cover the surface of the slant.

Use all sterile precautions in adding the phenol-saline solution to the culture medium to prevent access of spore formers.

Using a sterilized platinum or nichrome loop, loosen the growth from the surface of the slants, so that the living bacteria are suspended in the phenol-saline solution.

Flame the mouth of the test tubes and of the ½ ounce wide-mouth bottle.

Pour the suspension of living bacteria into the bottle.

Replace the cork, and hold in place with adhesive tape.

Shake in the shaking machine vigorously for at least five minutes, or shake by hand for fifteen to thirty minutes, or until all clumps of bacteria have been broken, and the bacteria are suspended evenly throughout the solution.

Sterilization.—

Place the suspension of bacteria in a water-bath regulated at 60° to 70° C. for one to three hours. Too little heat will fail to kill the bacteria, and too much heat may destroy the efficiency of the vaccine.

Sterility Test.—

Place the suspension of bacteria in the refrigerator after making the sterility test. Transfer several loopfuls of the suspension to two tubes of the type of culture medium used in the original cultures.

Incubate one of these aerobically, and the other anaerobically for three to ten days at a temperature of 37° C. and also use formula M-47, M-48, or M-49.

If no growth occurs at the end of this time, the vaccine is sterile, and may be diluted. If a growth appears, the vaccine must be placed in a water-bath at 80° C. for one or two hours, and another sterility test made at the end of this time.

The vaccine cannot be used unless it is sterile.

Counting (Wright's Method).—

Draw out a long capillary pipette in the flame.

Place a wax pencil mark at the end of every inch, beginning at the tip. The tip must be broken off squarely.

Have ready several clean glass slides, and cover glasses.

Have, also, a clean watch glass, on which are placed several drops of 2.5% sodium citrate solution.

Have ready a sterile puncture point used in making a blood count.

Work quickly.

Shake the contents of the vaccine bottle thoroughly.

Using a sterile dropper, withdraw several drops of the vaccine and place on a sterile watch glass. Use all sterile precautions, so as not to contaminate the vaccine.

Puncture the finger with the puncture point. The worker's own blood may be used.

Draw into the pipette 2 volumes of sodium citrate solution.

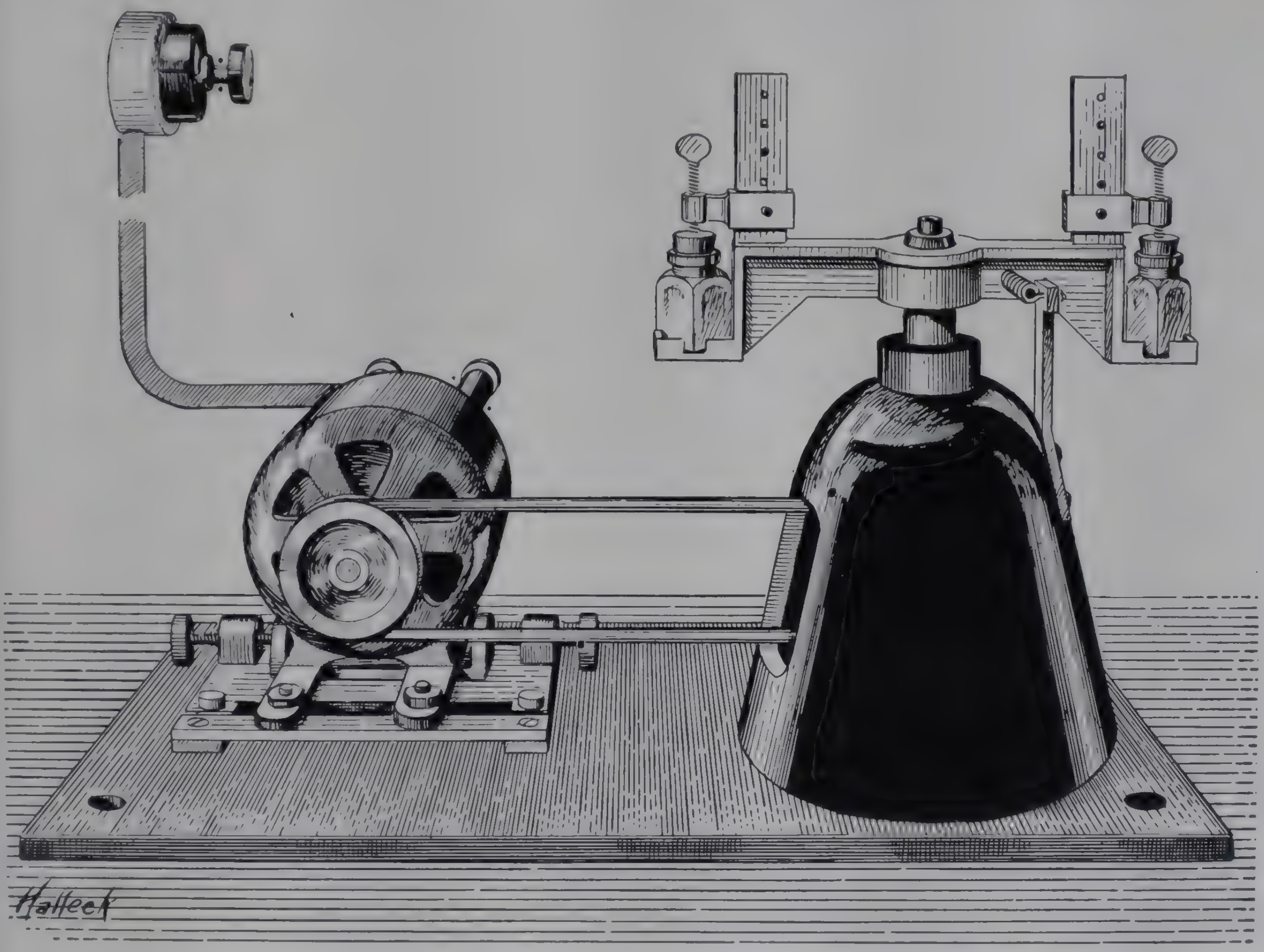

Fig. 346.—Shaking machine used in the manufacture of vaccines.

Admit a bubble of air after the two volumes of citrate solution.

Draw up 1 volume of blood.

Admit a bubble of air.

Draw up 1 volume of bacterial suspension.

Blow out upon a glass slide.

Mix thoroughly by sucking in and out of the pipette, taking care to avoid air bubbles.

Distribute to several slides.

Draw out a film in the manner of making a blood film.

Allow to dry.

Fix in methyl alcohol for five minutes.

Stain with dilute Giemsa stain for thirty to forty-five minutes, or stain immediately without fixation with Wright stain, page 620.

Use the oil immersion objective.

Count and record all the red blood cells and bacteria in successive fields, until 500 red blood cells have been counted.

Calculation.—

We know the number of red blood cells in a c.c. of blood. By making a comparison of the number of bacteria in a volume of vaccine equal to a volume of blood containing 500 red blood cells, we can compute the number of bacteria in a c.c. of the original vaccine.

Use the following formula:

Number of bacteria counted: X : : 500 : 5,000,000,000 (5 billion).

X equals the total number of bacteria in 1 c.c. suspension.

Example:

There were 750 bacteria counted in the fields in which there was a total of 500 red blood cells counted.

750 : x : : 500 : 5,000,000,000.

500 x equals 3,750,000,000,000

x equals 3,750,000,000,000 divided by 500 or 7,500,000,000 (7½ billion) bacteria in 1 c.c. of vaccine.

Diluting.—

The final vaccine suspension often contains 200,000,000 bacteria per c.c. (This number may be varied to suit individual cases.)

The vaccine in its present state contains 7½ billion bacteria per c.c.

The vaccine must be diluted so that each c.c. contains the correct number of bacteria, or 200,000,000.

Divide 7,500,000,000 by 200,000,000 to obtain the amount of dilution.

7,500,000,000 divided by 200,000,000 is 37.5.

Each c.c. of the present vaccine suspension must be diluted to a volume of 37.5 c.c. to obtain a bacterial count of 200,000,000.

Using all sterile precautions, place 1, 2, or 3 c.c. of the suspension (depending on the total volume desired after dilution) in a sterile graduated cylinder.

By means of a sterile 10 c.c. syringe, or a sterile pipette (20 c.c.), add the phenol-saline solution to the bacterial suspension until the desired volume is obtained.

Mix, and ampule.

Ampuling the vaccine.

Using a sterile syringe, and taking care to avoid air or other contamination, place the vaccine in ampules in 1 c.c., 2 c.c., or 5 c.c. quantities, as desired. The vaccine may also be placed in serum bottles, 20 c.c. in each vial.

Seal the ampules hermetically in the flame, and test for leakage after cooling, by shaking the contents thoroughly, holding the ampule upside down while shaking.

The serum vial is stoppered with a rubber stopper specially constructed to allow puncturing with a syringe needle when the contents are to be withdrawn. These rubber stoppers are sterilized in the autoclave at 15 pounds pressure for thirty minutes before using.

About 25 c.c. of the remainder of the vaccine are stored in serum vials so as to be on hand in case a refill is needed.

The vaccine should be labeled with the strain of bacteria present, the number of bacteria per c.c., the lesion from which they were obtained, the diluent, the patient's name, and the name of the physician.

For example:

Name of Patient Name of Physician.

Each c.c. of this vaccine contains 200,000,000 killed *Staphylococcus aureus* (suspended in phenol-saline solution) derived from abscess on forearm.

INOCULATION OF EXPERIMENTAL ANIMALS FOR CLINICAL DIAGNOSTIC PURPOSES

The use of experimental animals is necessary to verify certain bacteriologic procedures. The animals of choice are white mice, white rats, guinea pigs, and rabbits. It is necessary to procure healthy animals. For this purpose, one must be sure to buy his animal stock from reliable animal dealers with assurance that the animals have not been previously used by other laboratories for experimental purposes.

The animals must be kept in cages which can be readily cleaned. Provision must be made for the escape of urine from the bottom of the cage through the use of porous material or actual troughs.

Mice.—Mice may be fed on white bread and oats. Drinking water may be supplied through the special drinking apparatus which was introduced by Ehrlich and modified by Dunker.

Guinea Pigs.—The guinea pigs must be kept in dry roomy cages preferably with the bottoms covered with straw. They should be protected against sudden changes in temperature conditions. While guinea pigs were originally tropical or subtropical animals, in their changed location they stand the winter cold very well. They should be fed in summer on grass and pieces of vegetables, particularly cabbage. In winter, they may be fed hay and oats. Boiled potatoes are also good. Care must be exercised in changing their food supply in the spring and fall because of the likelihood of intestinal irritation. Artificially prepared foods can now be obtained for mice, guinea pigs, and rabbits.* A moderate amount of green vegetables, cabbage, etc., should also be given. When guinea pigs are being fed on cabbage or vegetables in summer it is well to give them very little water, but when they are given dry food they should be freely supplied with water.

Rabbits should be kept in roomy cages with plenty of straw. Their food requirements are about the same as for guinea pigs.

Cages of all experimental animals must be kept clean and disinfected at intervals.

Injection of Animals.—For the injection of animals, one must have on hand clean sterilized 2 c.c. glass syringes and 23-gauge 1-inch needles. To inject white mice, seize them at the nape of the neck and lay them on their backs, holding the tail or hind legs so they are stretched out and immovable. Subcutaneous injections are made by simply lifting the skin, after sterilization with iodine, and inserting the needle through the skin, care being taken in working around the abdomen not to penetrate the abdominal wall. Guinea pigs may be held in the same manner. For intravenous work on rabbits, select a marginal vein in the ear. The rabbit is placed upon a table, an assistant holds him securely while one or the other ear is selected for the injection. With a pair of fine scissors, the hair along the marginal vein is clipped off, the skin disinfected with bichloride of mercury or a small dab of iodine, the needle thrust into the marginal vein, and injection made.

For intra-abdominal or intraperitoneal injections, the rabbits or guinea pigs are secured on a special board, the abdominal wall lifted by the fingers so that no part of the intestine comes along with it, and injection is made with a needle in such manner as to avoid puncture of the intestines.

After the inoculation, the instruments used should be sterilized. The animals should be marked. Mice may be marked by placing a drop or two of various colored aniline dyes on their fur. Guinea pigs and rabbits may be marked by thrusting a very fine staple through the ear holding the numbered tag. These numbered tags may be bought from any supply house. For information on holding of animals, see pages 1494, 2041, and 2042.

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*Purina Mills, St. Louis, Mo., manufacture excellent feed or chow for laboratory animals.

The date of inoculation, number of tag, material, etc., should be entered in a notebook. The animal should be observed at frequent intervals. Notes should be made as to any signs of sickness. When animals die, they must be autopsied. Mice are autopsied by being placed on a board and tacked down with a tack through each extended extremity. Use sterilized instruments. With a pair of fine scissors the median incision is made and the visceral conditions are observed. Bacteriologic cultures sometimes must be made from autopsies of animals. These must be taken under strict aseptic conditions. The same method may be applied in autopsies on rabbits and guinea pigs.

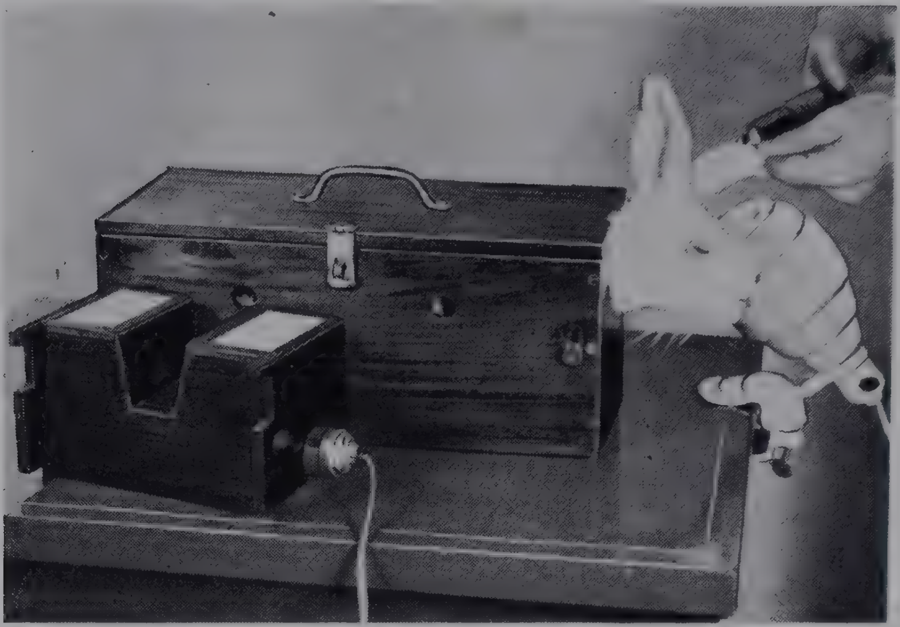

Fig. 347.—Beohm device for injecting into the marginal ear vein of a rabbit.

A very ingenious method for holding a rabbit so as to facilitate the injection of solutions into the marginal ear veins was described by E. B. Beohm.* This permits one to see that the needle is securely in the vein and that the blood is being pushed back through the venous system and not going out into the tissues. It consists of a light unit attached to a rabbit box, or a spotlight bed lamp which also works very well. This can be clamped to the edge of the table so that the rabbit's ear can be held out over the lens. Fig. 347 shows this type of lamp in use.

Another device is described in the chapter on Serology, pages 2041, 2042.

THE PRESERVATION OF BACTERIAL CULTURES

Maintaining a large collection of stock cultures of bacteria is a serious and difficult undertaking. Teaching institutions and type collecting agencies are confronted with this task which requires time, materials, and effort. It may involve the possible loss of biologic, immunologic, and cultural characteristics and occasionally the loss of the strain through such agencies as contamination, drying of the medium, and contact with accumulating metabolites. A voluminous literature has grown up on this question. Morton and Pulaski[1] worked out a method of preservation under paraffin oil which they claim has many distinct advantages. It reduces the frequency of contamination, especially with molds. No preliminary treatment of the cultures is necessary. All the organisms tested lived longer under oil than in the control tubes. Changes in cultural and biochemical characteristics have not been observed. The cultures are available at all times for transplantation without interfering with the preservation of the stock culture. The method is applicable to single colonies or mass cultures. It is advantageous in working with unstable variants, where occasional transferring to fresh media or growth in mass culture results in a change in the developmental stage of the strain. No seals, such as rubber caps, waxes, cements, etc., are needed. No special apparatus is needed, such as a centrifuge, desiccator, or vacuum pump. The method of choice recommended by these workers is as follows:

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*J. Lab. & Clin. Med. 26: 12, 1936-1937.

[1] J. Bact. 35: 163, 1938.

Organisms are grown on appropriate solid media, such as infusion agar, blood agar, etc., slanted so as not to give too long a slant. After good growth has taken place, the slant is covered with sterile heavy paraffin oil, or mineral oil, to a height of 1 cm. above the top of the slanted surface.

Attention must be called to the following points: The oil should be well above the uppermost level of the medium. The medium in this way cannot dry out and separate from the wall of the tube and float to the surface of the oil, in which event the organisms are usually found dead. The quality of the oil is very important as any rancidity or toxic substance is harmful to the organisms. They have found that mineral oil of Parke, Davis and Company has proved very satisfactory. It is preferable to sterilize the oil in the dry heat oven at 150° to 170° C. for one hour; during autoclaving moisture becomes mixed with the oil, giving it a milky appearance. Precaution is necessary in flaming the loop after it has been withdrawn from the oil, since plunging directly into the flame results in spattering. This may be prevented by warming the loop very gently before heating to redness, or by plunging into a beaker of boiling water, then flaming to redness in the usual manner. In our laboratories we have kept cultures alive for six months with this method. It is our method of choice.

Difco stock agar formula M-115 and BBL cystine trypticase base M-6 are excellent media for maintaining cultures of fastidious organisms.

Molds are grown on dextrose-free media.

Salmonellae are kept best in semisolid agar-gelatin M-84.

Vi cultures of Salmonellae are kept on Kauffmann's egg medium M-58c. These cultures are kept in the refrigerator and never left at room temperature except for a few minutes necessary for transfers. After transplanting, put the egg slants with the new inoculum in the incubator for 24 hours, then transfer them directly to the refrigerator.

Dehydration in vacuum, however, should be practiced whenever possible. Some organisms do not tolerate lyophilization well.

CLASSIFICATION OF THE PATHOGENIC BACTERIA

For complete classification of bacteria, refer to the most recent edition of D. H. Bergey, *Manual of Determinative Bacteriology*, Baltimore, The Williams and Wilkins Company.

Smooth and Rough Colonies

Repeated transplants of cultures often bring about loss of virulence of bacteria and frequently changes in their cultural characteristics.

The colonies on solid media become rough and dry, and in broth produce a characteristic granular deposit. The usual or ordinary form of bacterial colony is termed ''smooth''; when cultural changes in association with loss of virulence are present, the colony is termed ''rough.'' This change from smooth to rough is usually represented by the symbol S → R. A single species of bacterium can give rise to both these two distinct types of colonies. Roughness is in some cases related to loss of the complex carbohydrate associated with the capsule of the organism. Some bacteria are roughish in appearance even in their normal virulent form; for example, anthrax bacilli and hemolytic streptococci. This is due to the physical arrangement of the bacteria.

The change S → R is accompanied by a definite loss of serologic specificity and often by a definite loss of virulence. Hemolytic streptococci are an exception to the general rule, since the colony given by the normal virulent form is finely granular, not smooth, and the virulent form, which rises from it and has lost its type-specific antigen, is smoother than the normal form. When making vaccines of streptococci, rough colonies should be chosen since they are more antigenic than smooth colonies. Four different antigenic factors are involved with the typhoid bacillus. Failure always to use smooth colonies containing the complete antigen may explain why older work with vaccines was unsuccessful. This explains many irregularities in agglutination and other immunologic reactions.

The S-type colony of one species of bacteria may be regular, dome-shaped, homogeneous, and have a smoothly curved edge; the R-type colony has a roughened surface, granular consistency, sharply angular edge, and is larger and flatter than the S-type. There are many intermediate types. Smoothness and roughness vary with the species. One type of colony may arise from the other, but the production of S-forms from R colonies is the more difficult. Reliable stable types can be maintained almost indefinitely by repeated colony selection. The S → R change is easily made by the pneumococcus with loss of virulence, and antigenic and cultural changes.

The complete antigen of a virulent organism is made up of protein and carbohydrate, the latter being a partial antigen responsible for the type-specificity. The R-type has lost its ability to produce the carbohydrate portion; it, therefore, is not antigenic (type-specific). An R-organism can be converted into an S-form of another type by appropriate methods. Changes S → R and R → S can take place in the animal body and in the test tube.

Characteristics of smooth or S-type: Soft butyrous growth on agar; homogeneous clouding of broth and homogeneous suspensions in physiologic saline; active motility of the motile species; specific carbohydrate and capsules in the capsulated species; normal size and shape of cells; marked biochemical activities; somatic and flagellar or capsular antigens; coarse flocculation with antisera; usually virulent and toxic if a pathogen (although some streptococci are more virulent in the R form); associated with acute infections; sensitive to bacteriophage; resistant to phagocytosis; efficient immunizing agents.

Characteristics of rough or R-type: Granular, tenacious, or friable growth on agar; granular growth in broth; spontaneous agglutination in saline; usually lack flagella in the motile species and capsules in the capsulated species; cells vary in size, often filamentous with branching and fungus-like growth; reduced biochemical activities; somatic or group antigens only; usually lack virulence and ability to produce toxins; more common in chronic infections and in convalescence; less sensitive to bacteriophage; susceptible to phagocytosis; usually poor immunizing agent.

Gram-positive rods, such as *B. anthracis* and many clostridia, are pathogenic, and have their typical biochemical properties only in the rough form.

DESCRIPTION OF INDIVIDUAL BACTERIA[1]

Staphylococci (Micrococci)

Morphology: Spheres, 0.8 to 1.0 micron, occurring singly, in pairs, but more commonly in bunches which resemble bunches of grapes. They form a design-like arrangement in smears from solid cultures.

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[1] Revised from Bergey's Manual of Determinative Bacteriology, Williams & Wilkins Co., 1948.

Stain: Gram-positive.

Colonies: Growth abundant, colonies confluent, circular with unbroken edge, shiny, opaque, pigmented. They ferment lactose, sucrose, mannitol with acid formation.

Staphylococcus aureus forms a cream to golden orange pigment.

Staphylococcus albus and **epidermidis** form a white pigment.

Staphylococcus citreus forms a lemon yellow pigment.

Staphylococci cause localized abscesses; such as, boils, carbuncles, furuncles, etc. They are found in tonsillitis, osteomyelitis, etc.

While many strains forming orange pigment are pathogenic, the number of organisms producing white colonies and equipped with pathogenic power is less. It is necessary, therefore to subject staphylococci isolated from lesions to further tests:

(a) Hemolysin.—Two types of lysin are found: the alpha-lysin which causes the appearance of a hemolytic zone around colonies on blood agar formula M-28 or M-29 after 24 hours' incubation at 37° C.; and the beta-lysin which cause hemolysis on plates incubated for 24 hours at 37° C., and then placed for 24 hours in the refrigerator.

(b) Coagulase.—Test according to R-4. Most pathogenic staphylococci produce coagulase.

(c) Fermentation of Mannitol.—Many pathogenic staphylococci form acid from mannitol when tested in medium formula M-45.

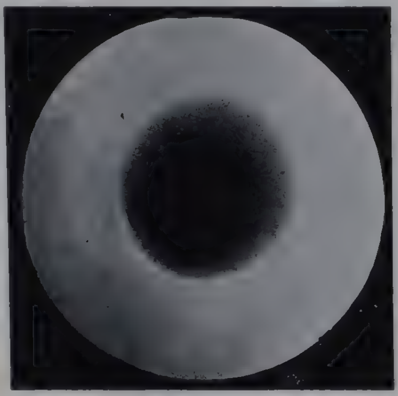

Fig. 348.

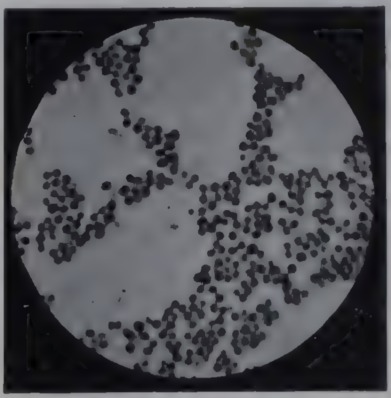

Fig. 349.

Fig. 348.—Colony of *Staphylococcus albus* on dextrose agar.
Fig. 349.—*Staphylococcus aureus*. (×950.)

(d) Liquefaction of Gelatin.—This is determined by inoculation of medium M-84 according to technic R-7, and on Stone's medium formula M-46.

Food poisoning staphylococci are subjected to the cat test which is described on page 1689.

For isolation of pathogenic staphylococci use blood agar plates formula M-28 or M-29. Excellent results are obtained with phenol red mannitol and salt agar M-45. This plate, described by Chapman,* may be used when only staphylococci are to be isolated; for example, in cases of intestinal upsets, from suspected food. Stone-Chapman medium (M-46) serves for further differentiation. The determination of hemolysis and coagulase confirms the diagnosis of a pathogenic staphylococcus.

For the detection of staphylococci in heavily contaminated material, plating to blood agar plates M-28 or M-29 is not always satisfactory. It may be necessary to use media with sodium azide (M-21) or phenylethyl alcohol (M-23). Food for examination should be ground in broth (M-1), then plated to blood agar M-28 or M-29 containing 3% sodium chloride, phenol red mannitol agar (M-45), and Chapman-Stone medium (M-46). Colonies showing mannitol fermentation are tested for coagulase formation (R-4), especially when a zone around them on the Chapman-Stone agar shows that they liquefy gelatin.

Serology.—This is rarely used for routine diagnosis. With the aid of precipitation tests, most staphylococci may be divided into two groups, A and B, the hemolytic types belonging mostly to group A. This classification of Julianelle was revised by Durfee[1] and extended. Fiske, and later McClure and Miller,[2] separated types of staphylococci by the bacteriophage method.

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*Chapman, G. H.: J. Bact. 48: 555, 1944.

[1] J. Bact. 44: 589, 1942

Agglutination and precipitation tests with the serum of the patient have no practical value.

Report.—It is suggested that *Staphylococcus albus* which does not form hemolysin or coagulase and has no action on gelatin or mannitol should be considered a contaminant. Instead of reporting *Staphylococcus aureus* as such an adjective ''hemolytic'' or ''coagulase-positive'' should be added.

Treatment with antibiotics and sulfonamides may change the biologic properties of staphylococci. The ability to produce pigment is lost first.

Sarcinae

Morphology: Spheres, varying in size from 1 to 2 microns. Arranged in packets showing three dimensions. The number of cells in each varies with the species.

Stain: Gram-positive, although they decolorize rapidly.

Colonies: Growth abundant, colonies confluent. They look like colonies of staphylococci.

Sarcina flava forms a lemon yellow pigment.

Sarcina aurantiaca forms a golden orange pigment.

Sarcinae are nonpathogenic.

Streptococci

Streptococci are divided into three groups, the grouping depending on their reaction with blood. **Streptococcus hemolyticus group** (including *Streptococcus pyogenes, scarlatinae, mixtus*, etc.) hemolyze blood.

Streptococcus viridans group (including *Streptococcus salivarius* [*mitior*], *faecalis*, etc.) form methemoglobin from hemoglobin.

Streptococcus nonhemolyticus group (including *Streptococcus anhemolyticus, saprophyticus*, etc.) do not react with blood.

Morphology: Spheres in chain formation, varying greatly in size, the cocci showing variations within the same and different chains. 0.5 to 1.0 micron in diameter.

Stain: Pathogenic streptococci stain gram-positive in neutral or alkaline media; gram-negative in acid media.

Many of the nonpathogenic varieties are gram-negative.

Colonies: Pinpoint, growth scant, colonies distinct. Show definite reaction with blood if they belong to either of the first two groups.

They are round, with lacelike, or slightly corrugated edges.

Macroscopically they are elevated and shiny.

Streptococci are the cause of many severe pus infections; they also cause erysipelas, scarlet fever, etc. They produce bacteriemia frequently. They are found in mastoiditis, infections of the bones, joints, serous cavities, etc.

Streptococcus pyogenes is the common streptococcus found in very many acute infectious processes, such as blood stream infections, mastoiditis, middle ear disease, cellulitis, infections of sinuses, bone and joint infections, metritis, and many others.

Streptococcus scarlatinae is the streptococcus found in the throats of scarlet fever patients, and thought to be the cause of scarlet fever.

Streptococcus puerperalis is found in general puerperal septicemia.

Streptococcus erysipelatos is the cause of erysipelas.

Streptococcus epidemicus is the cause of epidemic sore throat.

Streptococcus anginosus is found in sore throats.

Streptococcus stenos is found in many inflammatory conditions in man.

Streptococcus mixtus is found in many pyogenic inflammations.

Streptococcus infrequens is found in various human infections.

Streptococcus salivarius (mitior) is found in abscesses of teeth and in endocarditis.

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[2] Canad. M. A. J. 55: 36, 1946.

Streptococcus morbilli has frequently been found in blood, nose, throat, and conjunctiva of measles cases.

Streptococcus faecalis is found in the intestinal tract.

Streptococcus saprophyticus has been isolated from the vagina and from the nasopharynx in acute catarrhal inflammation.

Streptococcus anhemolyticus is a normal inhabitant of the mucous membranes of the mouth, nose, vagina, and intestines.

Many authors question the validity of the classification of streptococci according to their ecology, and suggest that hemolysis and serologic tests should be the chief means of differentiation.

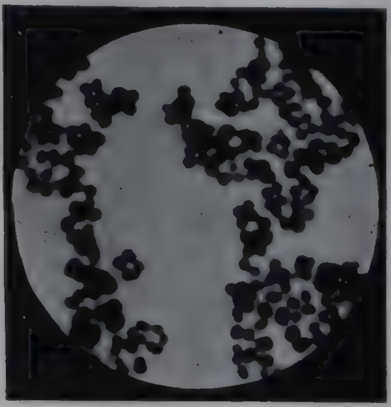

Fig. 350.

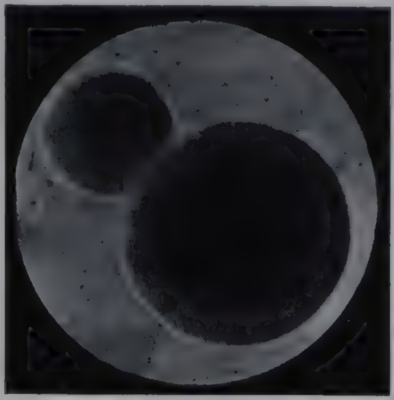

Fig. 351.

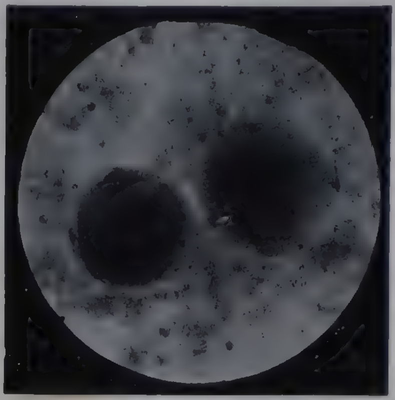

Fig. 352.

Fig. 350.—*Sarcina flava.* (×950.)

Fig. 351.—Colonies of *Streptococcus hemolyticus* on blood agar. Note that all the blood cells have been dissolved.

Fig. 352.—Colonies of *Streptococcus viridans* on blood agar. Note only partial hemolysis of blood cells.

Classification of Streptococci.—(See R-3.) Brown described two different types of reaction in blood agar plates, as follows:

α. A green discoloration and partial hemolysis of the blood corpuscles, forming a rather indefinitely bounded zone, 1 to 2 mm. in diameter, outside of which is a second, narrow, clearer, not discolored zone. Very few corpuscles remain in the outer, clearer zone, and these are never discolored. Even after 48 hours' incubation, this narrow outer zone of hemolysis does not always develop. If a plate, which has developed the typical appearances, is reincubated 24 to 48 hours, and then placed in the refrigerator for a further 24 hours, a double series of rings will develop frequently, so that the colony is surrounded by a hazy discolored ring, a clear hemolyzed ring, a second hazy ring, and a second clear ring.

β. These colonies are surrounded by sharply defined, clear, colorless zones of hemolysis, 2 to 4 mm. in diameter. No corpuscles can be seen within this zone. These zones develop more rapidly than those of the alpha type, often seen after 18 hours' incubation. They undergo no change in the subsequent 24 hours in the icebox.

The β-hemolytic strains just described correspond to the old classification of Schottmüller, called *Streptococcus hemolyticus.* The α-hemolytic strains may be regarded as equivalent to his *Str. viridans.* In bacteriologic circles, "hemolytic streptococcus" really means a strain that causes β-hemolysis on blood agar; a "nonhemolytic streptococcus" means a strain that either produces α-hemolysis or gives rise to no change at all.

Fermentation Reactions of Streptococci.—

Fermentation reactions are now employed as auxiliary rather than as primary differential criteria. Streptococci isolated from pathogenic lesions in man usually ferment lactose and salicin, but seldom mannitol, raffinose, or inulin, and produce acid without clot in milk. Streptococci commonly found in the human mouth, which are of the α-hemolytic type, frequently ferment raffinose, but fail to ferment salicin, inulin, and mannitol, and produce coagulation in milk. The fermentation tests must be carried out in serum broth (M-7), or cystine-trypticase base with the fermentable substances (M-6).

The Antigenic Structure of Streptococci.—

This problem has been well studied and worked out by Lancefield, who prepared extracts from different strains of streptococci and tested them against homologous and heterologous antisera by means of the precipitin reaction tests. She differentiated three different antigenic components. One of these antigens is an acid-soluble protein. Lancefield names this the "M substance." Another component is a complex carbohydrate found in hemolytic streptococci. It is analogous to the noncapsular carbohydrate component that characterizes the species *Str. pneumoniae*. She divided various strains of streptococci into groups. Twenty-one out of 23 strains possessing the polysaccharide antigen were called Lancefield's Group A and were derived from human sources, mainly from cases of acute infection; 18 of 21 strains belong to Group B, derived from cattle, mainly from cases of mastitis; 49 strains belong to Group C, derived from various animals, guinea-pig, cow, rabbit, horse, fox, pig, and fowl, etc., all suffering from definite infections; 8 strains belonged to a fourth group, D, derived from cheese; and 3 strains belonged to a fifth group, E, all isolated from certified milk.

Group A has been further differentiated into a number of different types by the type-specific, acid-soluble protein components. Group B is also divided into several antigenic types. There also have been added to this classification groups F, G, H, K, L, and M.

Group A.—These streptococci contained the Group A carbohydrate component described by Lancefield. They can be divided into a number of different serologic types by agglutination reactions or by precipitin reactions. Group A streptococci, in addition to producing β hemolysis in blood agar plates, form a soluble hemolysin. They produce a final pH of 5.0-5.6 in dextrose broth; they do not hydrolyze sodium hippurate; they do not reduce methylene blue in milk; they ferment trehalose but not sorbitol; they may or may not grow in 10 per cent bile agar, and seldom grow in 40 per cent bile agar. They ferment salicin, lactose, seldom mannitol, and very seldom raffinose or inulin. The strains that have been adequately identified as belonging to this group have, in the main, been derived from infections in man—tonsillitis, scarlet fever, cellulitis, erysipelas, puerperal fever, other types of septicemia, acute bronchopneumonia, otitis media, and so on. They have also been found in the throat, nasopharynx, or nose, of normal persons.

Group B.—These streptococci share in common a group-specific antigen that is carbohydrate in nature and differs from the group-specific antigen of Group A. They are divided further into a number of antigenic types, of which three have so far been identified, either by precipitation or agglutination tests against type-specific antisera. The type-specific antigens within this group are apparently not acid-soluble proteins, but complex carbohydrates of a different chemical structure from the carbohydrate that determines group-specificity. They may or may not produce β-hemolysis in blood agar plates. They differ from Group A strains in that they produce a lower final pH in glucose broth, hydrolyze sodium hippurate, and usually grow both on 10 per cent and 40 per cent bile agar. Like Group A strains, they do not reduce methylene blue in milk, but they resemble Group A in fermenting trehalose but not sorbitol. Most Group B strains have been isolated from cases of mastitis in cattle but occasionally they have been isolated from the normal human throat and vagina.

Group C.—These strains share in common a group-specific antigen that differentiates them from the other groups. They produce β-hemolysis on blood agar plates, and form a filtrable hemolysin. They produce in glucose broth a final pH intermediate between that produced by Group A strains on the one hand and by Group B strains on the other; the range covered by this group varies between pH 4.5 and pH 5.4. They do not hydrolyze sodium hippurate. Most strains of Group C grow on 10 per cent bile agar, but very few on

40 per cent bile agar. They can be further differentiated into three subgroups. One of these subgroups ferments neither trehalose nor sorbitol, another ferments sorbitol but not trehalose, and a third ferments trehalose but not sorbitol.

Group D.—This group, which was differentiated by Lancefield, possesses a common group-specific antigen, the chemical nature of which has not yet been determined. They produce β-hemolysis on blood agar. They do not produce a filtrable hemolysin. They produce a low final pH in glucose broth, 4.2 to 4.8. They grow freely on 10 per cent and 40 per cent bile agar. They reduce methylene blue in milk. They are heat resistant, most strains withstanding a temperature of 60° C. for thirty minutes. They do not hydrolyze sodium hippurate, usually but not always ferment trehalose and sorbitol, always ferment lactose and salicin, and usually but not always mannitol. They tend to assume the diplococcal rather than the streptococcal grouping. They have been derived from cheese and human feces.

Group E.—These strains have been isolated from cow's milk by Lancefield.

Group F.—These possess a group-specific antigen, by means of which they may be identified. They grow slowly on blood agar plates, forming minute pinpoint transparent colonies surrounded by a narrow zone (1.5 to 1.8 mm. in diameter) of β-hemolysis. They are identical with the strains described by Long and Bliss as ''minute hemolytic streptococci.'' They produce a final acidity in glucose broth of pH 4.8 to 5.2. They do not hydrolyze sodium hippurate. They do not reduce methylene blue in milk. They do not grow either on 10 per cent or on 40 per cent bile agar. None ferment sorbitol but some strains ferment trehalose. They ferment lactose and salicin. This group has been found in cultures from the human throat and probably are responsible for some cases of tonsillitis.

Group G.—These strains share also in common a group-specific antigen that distinguishes them from any of the other named groups. There is a controversy as to the final pH produced by them in glucose broth. Lancefield and Hare give a pH of 4.6 to 5.2, but Plummer gives to them a pH of 4.4 to 4.6. They do not hydrolyze sodium hippurate. They grow on 10 per cent bile agar, but do not multiply on 40 per cent bile agar. They do not reduce methylene blue in milk. They ferment trehalose, but not sorbitol. They ferment lactose. Most of these strains have been isolated from man, monkey, or dog. They seldom cause very severe infections.

Groups H and K.—They appear to differ from each other and from the other groups of hemolytic streptococci, in regard to their group-specific antigens They have been isolated from the nose or throat of normal persons.

Groups L and M.—These groups have been established more recently. M group streptococci were found in dogs.

Alpha-Hemolytic Streptococci.—Alpha-hemolytic or ''viridans'' streptococci usually grow at 45° C. if of animal origin. The latter hydrolyze starch. *Streptococcus endocarditidis* is a member of the viridans group.

The most important member of the ''viridans'' group is the Streptococcus of subacute bacterial endocarditis *(Streptococcus s.b.e.)* described by Niven and White[1] as:

(a) alpha hemolysis; (b) arginine positive; (c) inulin positive (M-6 or M-7); (d) raffinose negative (M-6 or M-7); (e) no action on sodium hippurate R-11; (f) often growth on media containing 40% bile; (g) does not grow on 6.5% sodium chloride-containing-media (medium made from M-18); (h) produces viscous growth in broth containing 5% sucrose, because of dextran formation; (i) does not respond well to penicillin treatment.

Lactic Streptococci.—These grow well at 10° C. and reduce methylene blue. They are present in dairy products, and are considered nonpathogenic.

Enterococci.—These survive 60° C. for 30 minutes, grow in the presence of 6.5% sodium chloride, and in media with strongly alkaline reactions. Some of them can be grouped with Lancefield's serum ''D,'' and are hemolytic. They are usually found in the intestinal contents.

Micro-Aerophilic Streptococci.—These organisms are usually isolated from wounds, intestinal contents, genitourinary tract, and blood stream. Most of them are nonhemolytic or belong to the viridans type. Many aerobic streptococci become microaerophilic under the influence of penicillin treatment.

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[1] J. Bact. 51: 717, 1946.

Anaerobic Streptococci.—These are found in pus, genitourinary tract, blood stream (principally in puerperal sepsis and diseases of the lower abdomen). Most of them are strongly proteolytic. Their classification is vague.

In the study of **aerobic streptococci** the following reactions are of greatest importance:

(a) **Hemolysis.**—Streaked, poured, and poured-streaked blood agar plates M-28 or M-29 are used.

(b) **Inulin Fermentation.**—Most streptococci do not ferment inulin in formula M-6 or M-7.

(c) **Fermentation of Trehalose and Sorbitol.**—Many strains of group C and all strains of group K fail to ferment trehalose. Some strains of groups A, D, F, G, and H may be trehalose-negative. Sorbitol is fermented only by E strains and many C and D strains.

(d) **Hydrolysis of Sodium Hippurate.**—All strains of group B and many strains of group D produce hydrolysis of this compound. The reaction is tested according to R-11.

(e) **Fibrinolysin.**—It is determined with the aid of R-5.

The primary isolation of streptococci from uncontaminated material, such as blood, cerebrospinal fluid, etc., is carried out in trypticase soy broth (M-3), chopped meat medium (M-12), brain heart infusion broth (M-4), and on blood agar (M-28 or M-29). Microaerophilic as well as other streptococci grow well in thioglycollate broth (M-47, M-48).

For the isolation of enterococci, the S.F. enrichment medium of Hajna (M-112) is recommended.

Sodium azide blood plate (M-21) and phenylethyl alcohol plate (M-23) are excellent media for the isolation of streptococci from contaminated material; e.g., throat swabs. The sodium azide blood broth (M-19) is an excellent shipping medium for such material.

Fermentation is tested in broth M-5 with tryptose or trypticase, and in cystine trypticase medium M-6. One-half per cent of the fermentable substances is added to the base.

SEROLOGY

Griffith's slide agglutination test is difficult to carry out in routine laboratory practice because of the great number of cross-reactions. The precipitation technic is, therefore, preferred by many workers.

(a) **Production of Precipitating Sera.**—The antigen for all group sera, with the exception of group D, is prepared by growing the culture in buffered extract broth formula M-2 for 24 hours. Add enough 1% formolized saline to make the final formol concentration 0.05%. Store in the refrigerator for 48 hours. Make tests to determine if organisms are dead. If they are not, prolong the storage. Immunize rabbits directly from these suspensions. Group D streptococci must be centrifuged and resuspended in saline before being used for inoculation (Coffey).

Inject 1 c.c. amounts of these antigens into rabbits each day for 6 successive days. Remove some blood and test. If the test bleeding shows a high enough titer, bleed the rabbits 5 to 7 days after the last injection. Test the sera with homologous and heterologous streptococci for specificity and titer.

(b) **Preparation of Extract From Tested Organisms, Coffey Technic, Modified.**—Grow streptococci in trypticase soy broth formula M-3 for 24 hours. Use a large inoculum to start the growth. Incubate and centrifuge for one hour at 2500 to 3000 r.p.m. Decant the supernatant fluid and add saline to the sediment equal to $\frac{1}{5}$ the original volume of the broth. Add N/1 hydrochloric acid in amount equal to $\frac{1}{20}$ the volume of the saline-sediment mixture. Heat in the boiling water bath for 20 minutes. Transfer to the refrigerator. When cool, centrifuge and decant the supernatant fluid. Add to the supernatant fluid 1 drop of brom thymol blue indicator (page 32) and adjust the reaction to pH 7.0 to 7.2 with N/1 sodium hydroxide. Centrifuge again and use the supernatant fluid.

(c) **Another method (Lancefield)** recommends the use of the ''C'' substance: Add to the growth in buffered neopeptone broth M-2, after 18 hours' incubation, an equal amount of N/10 hydrochloric acid. Let stand 18 hours, then centrifuge. Heat the precipitate on the boiling water bath for 10 minutes, then cool and centrifuge. Decant the supernatant fluid and pour it into 4 volumes of 95% ethyl alcohol. Let stand for 24 hours, centrifuge, keep the sediment on the boiling water bath until the alcohol has completely volatilized off. Dissolve the sediment in an amount of saline equivalent to the volume of the original neopeptone broth.

(d) **Precipitation Test.**—

Prepare tubes 8 mm. in diameter, in rows, one row for each serum. For routine examination, three rows, corresponding to A, B, and C group sera, are used. Each row has six tubes.

Use the antigen undiluted, diluted 1:10, and diluted 1:100.

Tube	1	2	3	4	5	6
Serum	0.2	0.2	0.2	0.0	0.2	0.2
Antigen from unknown organism	0.2 undil.	0.2 dil. 1:10	0.2 dil. 1:100	0.2 undil.	0.0	0.0
Antigen from known specific organism	0.0	0.0	0.0	0.0	0.0	0.2 undil.
Saline	0.0	0.0	0.0	0.2	0.2	0.0

This table shows the set-up of each row. The controls are antigen control (tube 4), serum control (tube 5), and test with a homologous organism.

Incubate for 10 minutes in a water bath at 37° C. and determine if a ring has formed at the point of contact between the serum and the antigen. If no ring formation is observed, mix the contents and reincubate for 2 hours at 37° C. Observe for precipitation. If the reaction is still negative, keep the tubes in the refrigerator for 12 to 14 hours before taking the final reading.

Reliable commercial sera are produced by Lederle, Inc.

Determination of the Antistreptolysin Titer, Rantz and Randall[1]

(a) **Preparation of Lysin.**—Grow Richards strain, or any other strain of known good hemolysin producing streptococci belonging to group A, in meat infusion broth containing 0.2% dextrose, 2% proteose-peptone, and buffered by the addition, after autoclaving, of 0.2% sodium bicarbonate in sterile solution.

Centrifuge after 24 hours. To each 100 c.c. of the supernatant fluid add 42.9 gm. of ammonium sulphate, and let stand at 20° C. Centrifuge and dissolve the sediment in phosphate buffer pH 7.0, and make up to 1/25 of the original volume of the culture with the buffer solution. Dialyze in cellophane sacks for 18 hours against running tap water. Add 9 gm. sodium chloride to each liter of the dialyzed material, and sterilize by passing through a Seitz filter.

Phosphate buffer pH 7.0 is made by dissolving 14.2 gm. of disodium phosphate · 12 H_2O and 3.63 gm. of monopotassium phosphate in a liter of distilled water.

(b) **Determination of Combining Unit.**—Dissolve 1.6 gm. sodium hydroxide in 1000 c.c. of an isotonic buffer of pH 6.5.

Buffer pH 6.5 is made by dissolving 4.2 gm. sodium chloride, 3.17 gm. monopotassium phosphate, and 3.58 gm. of disodium phosphate · 12 H_2O per liter of solution.

Make a fresh solution of 0.15 gm. cysteine hydrochloride in 25 c.c. of this solvent.

Set up mixtures of the lysin and the buffered cystine:

c.c. lysin	0.35	0.30	0.25	0.20	0.15	0.10
c.c. cysteine	0.15	0.20	0.25	0.30	0.35	0.40

After 10 minutes add to each tube 1 c.c. of a standard antistreptolysin, containing one unit per c.c. Incubate for 15 minutes in the water bath at 37° C. Add to each tube 0.5 c.c. of a 5% suspension of rabbit blood cells suspended in the pH 6.5 buffer without sodium hydroxide. Incubate for 45 minutes. Centrifuge. That amount of lysin which just failed to produce hemolysis is the combining unit.

(c) **Determination of Antistreptolysin Titer.**—Dilute the concentrated lysin (b) to the degree determined in the previous test with isotonic buffer. A further dilution is carried out in the buffered cysteine solution at the time of use, so that the combining unit will be present in a final volume of 0.5 c.c. The lysin is ready for use after 10 minutes' exposure to the reducing agent (alkaline cysteine) and does not deteriorate within 60 minutes.

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[1] Proc. Soc. Exper. Biol. & Med. 59: 22, 1945.

Set up the reaction as follows:

SERUM DILUTIONS

	1:10		1:100					1:500				
C.c. diluted serum	0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2
C.c. isotonic buffer	0.2	0.8	0.0	0.2	0.4	0.6	0.7	0.0	0.2	0.4	0.6	0.8
C.c. reduced lysin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Unit per tube	12	50	100	125	166	250	333	500	625	833	1250	2500

Incubate in the water bath at 37° C. for 15 minutes. Add to each tube 0.5 c.c. of the buffered rabbit red blood cells. Incubate for an additional 45 minutes.

The last tube in which hemolysis has *not* occurred indicates the antistreptolysin titer of the serum.

A control serum of known titer should be included as a check.

More than 200 units is considered a pathologic finding.

ANIMAL INOCULATION

Mice are usually susceptible to pathogenic human streptococci.

Warning.—In cases treated with antibiotics the biology of the streptococci may be greatly changed. If experiments with hemolysin production on blood agar plates fail, use R-3 for the determination of this activity, preferably with a suspension of 5% sheep blood cells. Mix 0.5 c.c. of these cells with 0.5 c.c. of the broth culture and incubate for 2 hours at 37° C.

It is best to collect material from patients suspected of having streptococcus infections into broth containing penicillinase (M-20) *or* clarase and p-aminobenzoic acid, because treatment with penicillin or sulfonamides or both is often instituted before the specimens are collected.

SENSITIVITY TEST

The Dick Test for Susceptibility to Scarlet Fever

This test is based upon the assumption that the streptococcus is the cause of scarlatina. In 1885, Kline suggested that the streptococcus might be the cause of scarlet fever. In 1899, Class, of Chicago, and Gradwohl, of St. Louis, described an organism, a diplococcus, which was found in the throat of scarlet fever patients. With it, they were able to reproduce a febrile disease with desquamation in young hogs. In 1923, the Dicks, of Chicago, established facts which seemed to prove that the hemolytic streptococcus is responsible for scarlet fever. It is reasonable now to assume that the hemolytic streptococcus that is responsible for scarlet fever in one person may cause merely a harmless throat infection in another person. In England, Griffith worked out 28 different types of hemolytic streptococci. Types I and II were reported as predominant in London scarlet fever patients and other types were found elsewhere. In Chicago, Bailey found types II, III, and VI predominant in patients admitted to the Municipal Hospital in the years 1934 and 1935.

Some workers have attempted to prove that scarlet fever is due to a virus, but until more evidence is brought forward, it is proper to recognize the hemolytic streptococcus as the cause of this disease.

Purchase the Dick toxin for skin tests from any reliable biological house. This product is made so that 0.1 c.c. represents the quantity for injection.

Cleanse the flexor surface of the forearm with alcohol.

Using a 1 c.c. tuberculin syringe, inject intradermally exactly 0.1 c.c. of the Dick toxin.

With another syringe, inject 0.1 c.c. sterile physiologic salt solution about 2 inches below the test injection.

Needles should be the same as for the Schick test.

Wait twenty-four hours.

Read the reaction in a good light.

If the saline injection shows a reaction, disregard the result of the test.

If the saline injection shows no reddening, the test may be read.

Regard any reddening of the skin at the area of inoculation as a positive reaction

It is the custom of some observers to disregard any reaction of less than 1 cm. diameter.

A positive reaction indicates that the patient is susceptible to scarlet fever infection.

The Blanching Test for Diagnosis of Scarlet Fever

By injecting a small amount of scarlet fever **antitoxin** beneath the skin of the rash, a diagnosis of active scarlet fever infection can be made.

If the rash blanches at the point of inoculation, it is a true scarlet fever rash, not a drug rash.

Pneumococci

(Diplococcus Pneumoniae)

Morphology: Oval or spherical organisms, 0.6 to 0.8 micron by 1 micron. They may occur singly, in pairs, or in short chains, the chains showing the pair formation. In fresh specimens, and often in cultures, they show a capsule, which surrounds the single organism, or the pair, or the chain. They are lancet-shaped, and arranged along the long axis.

Stain: Gram-positive.

Colonies: Pinpoint, distinct colonies. Margin is entire; colonies are grayish. Blood agar shows these colonies with a zone of methemoglobin, indistinguishable from colonies of *Streptococcus mitis (viridans). Diplococcus pneumoniae* is soluble in bile and ferments inulin. *Str. viridans* is not bile soluble and does not ferment inulin.

Growth is scant on plain agar.

This organism is the common cause of lobar pneumonia and is associated with inflammations of the upper respiratory tract. There are now thirty-three recognized types of pneumococci differentiated by agglutination with specific serum or by the Neufeld reaction

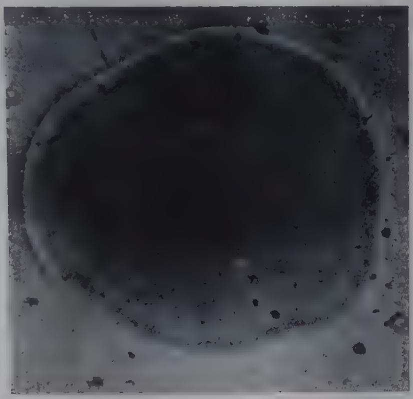


Fig. 353.—Twenty-four-hour colony of *Neisseria gonorrhoeae* on Gradwohl special hormone brom cresol purple medium.

Pneumococci must be tested for the following:

(a) **Hemolysis.**—Blood agar M-28 or M-29 or R-3 will show the alpha type of hemolysis.

(b) **Bile Solubility Test.**—

All pneumococci are bile soluble. Bile solubility may be tested with bile, sodium desoxycholate, or sodium lauryl sulphate. The method is described under R-10.

(c) **Inulin Fermentation.**—Test for inulin fermentation in M-6 or M-7.

Trypticase soy broth M-3 and brain heart infusion M-4 are recommended for the isolation of pneumococci from blood and cerebrospinal fluid. Material such as sputum is streaked on blood agar plates M-28 or M-29. The species-diagnosis is carried out by typing. This is useful only when the organisms have capsules. Capsules may be lost under the influence of therapy. It is necessary, therefore, to ascertain, by the use of capsular stains, if the organisms are typable.

The best enrichment is the mouse inoculation.

Mouse Injection for Use With Sputum Typing in Pneumonia

This method is used when the sputum fails to show the Quellung reaction in direct typing, and also to verify the type when the Quellung reaction has been found. If the sputum is very thick or tenacious, add a small amount of sterile saline or broth to facilitate ejection through the needle.

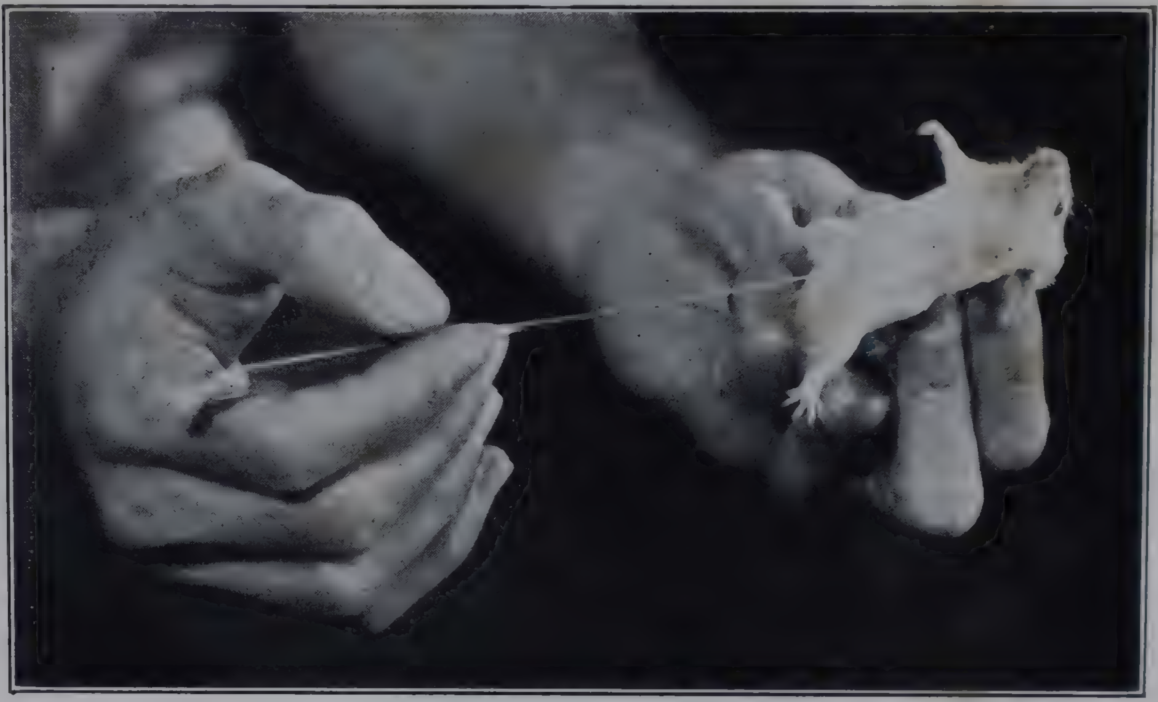


Fig. 354.—Correct method of holding a white mouse for intraperitoneal injection.

Pick up the mouse by the tail using the right hand. Then grasp the mouse by the nape of the neck with the thumb and index finger of the left hand. (This can be done more easily if the mouse is holding on to the edge of the table or wire of its cage with its front feet.) Maintaining this position, turn the mouse over, pulling its tail down the center of the palm toward the wrist. Hold the tail and left hind leg down with the little finger. With the middle and ring fingers, hold the loose skin of its back tightly. Locate the soft depression near the right hip of the mouse. Swab this area with iodine. Inject 1.0 to 1.5 c.c. of sputum intraperitoneally into the soft spot, using a syringe and intradermal needle (about ¼ inch long). After 4 to 6 hours, or sooner if the mouse appears ill, hold the mouse in the same position and tap the abdomen. Use a capillary-dropper with a fine yet not flexible end. Insert this into the abdomen of the mouse and withdraw fluid for typing.

SEROLOGY

Typing of Pneumococci

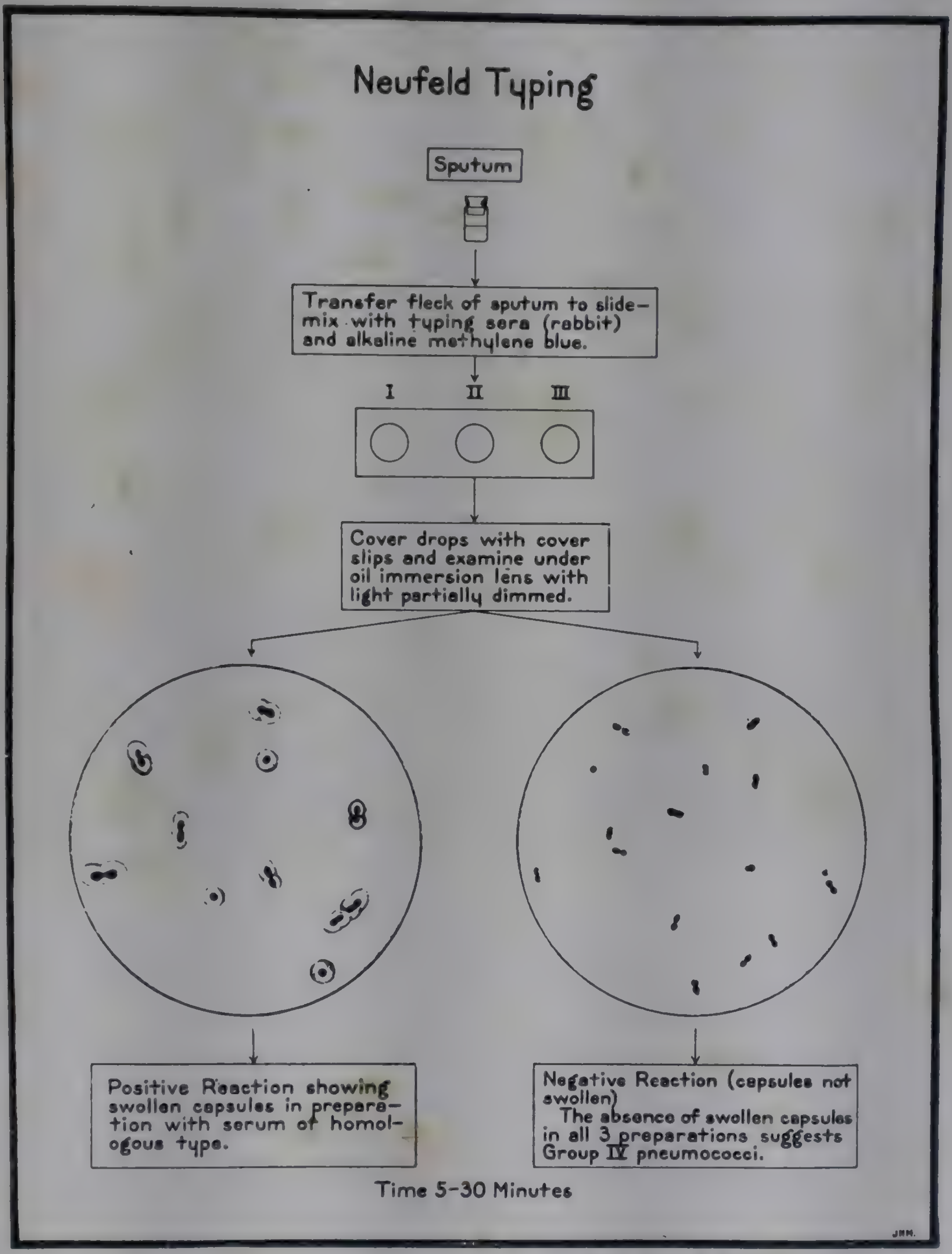
The Neufeld ''Quellung'' Reaction

This is the method of choice for quickly and accurately typing pneumococci. It was first described by Neufeld and later modified by Sabin.

The word ''Quellung'' means the swelling of the capsule of the pneumococcus when it comes in contact with its homologous serum.

Technic.—The typing sera may be obtained from Wyeth, Inc., Philadelphia.

There are six pooled sera available: mixture A, containing Types I, II, and VII; mixture B, containing Types III, IV, V, VI, and VIII; mixture C, containing Types IX, XII, XIV, XV, XVII, and XXXIII; mixture D, containing Types X, XI, XIII, XX, XXII, and XXIV; mixture E, containing Types XVI, XVIII, XIX, XXI, and XXVIII; and mixture F, containing Types XXIII, XXV, XXVII, XXIX, XXXI, and XXXII.


Neufeld Typing
Sputum
Transfer fleck of sputum to slide—mix with typing sera (rabbit) and alkaline methylene blue.
I
II
III
Cover drops with cover slips and examine under oil immersion lens with light partially dimmed.
Positive Reaction showing swollen capsules in preparation with serum of homologous type.
Negative Reaction (capsules not swollen)
The absence of swollen capsules in all 3 preparations suggests Group IV pneumococci.
Time 5-30 Minutes
JRH.

Mass. Dept. of Public Health.

Fig. 355.—Schematic representation of steps in Neufeld typing together with a positive and negative reaction.

Using a platinum* loop, place a small fleck of sputum on each of six cover slips. Add to each bit of sputum 2 or 3 times the quantity of the various mixtures, using a different pooled serum for each cover glass. Add two loopfuls of Loeffler's methylene blue solution to each bit of sputum. Mix thoroughly with a platinum loop and invert on ordinary glass slides, pressing them down to make a thin layer of stained sputum between the cover glass and the slide. Each slide must be properly labeled. Examine the preparation with the oil immersion objective with the diaphragm partially closed. Make a note of which mixtures show swelling of the capsules of the pneumococci. When a mixture is found which gives a positive reaction, the sputum is then tested against monovalent sera for each type included in the mixture.

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*On nichrome.

For example, capsule swelling occurred in mixture B, which contains Types III, IV, V, VI, and VIII. Prepare five coverslips with sputum on each and then add 2 or 3 times the quantity of Types III, IV, V, VI, and VIII to the respective cover glass. Mix with a platinum loop, invert over a glass slide, press down, and examine with the oil immersion objective. Report the types which show swelling of the capsules. If the preparation does not contain enough methylene blue, a small amount may be added with the platinum wire.

A positive reaction is indicated by the appearance of definitely outlined capsules about the pneumococci present in the sputum. The sharpness of the outline of the capsule is perhaps of more diagnostic importance than the degree of swelling. A positive reaction usually takes place within a few minutes. If no reaction occurs immediately, the preparation may be examined again at the end of 30 minutes. In pneumococci with negative reaction, the capsule will appear as a halo without definite outline. Sabin stated that the reaction is so specific that the type diagnosis may be made if only one swollen capsule is seen in the preparation. A positive reaction usually takes place within a few minutes.

When the Neufeld technic is used with sputum containing many Type III organisms, the organisms may appear in large masses without definite capsular swelling. In such cases, it may be necessary to dilute the sputum with saline to obtain definite swelling of the capsule.

Sputum, if kept on ice, may be typed quite satisfactorily even when several days old. The method is also applicable to cultures, spinal fluid, pus, etc.

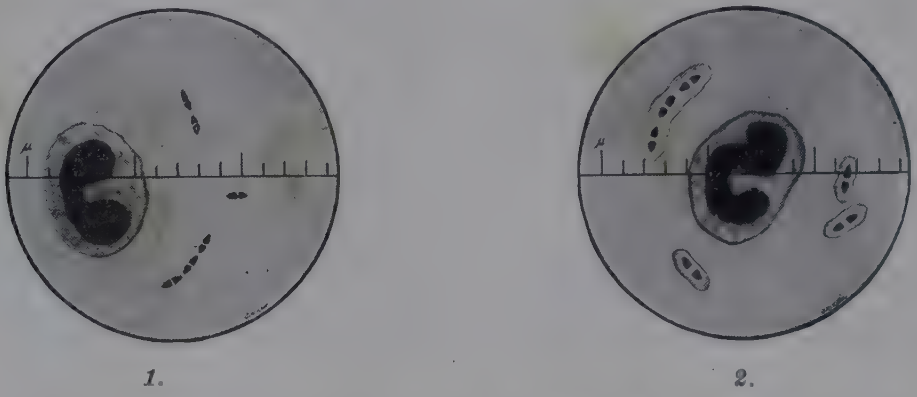

Fig. 356.—Neufeld reaction showing, *1*, negative and, *2*, positive results. Note the size of the swollen capsules on *2* as compared with the leukocyte. (Courtesy Dr. A. B. Sabin and J. A. M. A.)

Sabin or "Stained Slide" Agglutination Test[1]

The mouse peritoneum is injected with 1 or 1.5 c.c. of sputum, and after three or four hours a glass capillary is filled with peritoneal fluid directly through the abdominal wall. Capillary drops of fluid are expelled onto a slide and mixed with the different typing sera either individually or in groups. The one in which pneumococci become clumped is the one in which serum and organisms are the same. The others serve as controls.

If there is no clumping, the type or group of types may be excluded; if there is clumping in a group test, the sera of the types contained in it are separately applied until the type which causes the clumping is found. A bile solubility test should be done on the broth culture of the organisms (R-10).

Krumwiede and Valentine Precipitation Test

Filtrates of sputum, cerebrospinal fluid, urine, and pleural exudate may contain pneumococcus precipitinogen in the absence of the organisms because of autolysis of the bacteria. Such substances may be found also in the peritoneal cavity of mice.

The fluid suspected of containing pneumococcus precipitinogen is used for precipitation reactions in the same manner as streptococcal extracts described on page 1490.

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[1] Sabin, A. B.: Proc. Soc. Exper. Biol. & Med. 26: 492, 1929.

NEISSERIAE

Neisseria Gonorrhoeae (Gonococcus)

CULTURE METHODS

Ever since the publication of the work of Neisser in 1879 on the gonococcus, and its cultivation by Bumm in 1885 on human blood serum, the exactitude of bacteriologic gonococcal diagnosis has been taken too much for granted. There has been too much reliance placed on the Gram stain as a definite means of identification. This stain shows many gram-negative diplococci which are not gonococci, but which resemble them morphologically. The variability of the gram reaction, as reported by the Committee of Bacteriological Technic, of the Society of American Bacteriologists[1] is well known among bacteriologists, but practitioners have been altogether too confident concerning the reliability and infallibility of the Gram stain. Churchmann[2] showed that with some organisms the Gram reaction is ''stable,'' and with others ''unstable.'' Stearn and Stearn[3] showed that to obtain a successful differentiation between positives and negatives, the Gram technic should be performed at a controlled reaction of about pH 4.0. While the Committee report showed that the Gram technic is more useful in diagnostic work than it is in pure culture work on unknown bacteria, we believe some of its fallacies and shortcomings may be operative in the field of gynecologic bacteriologic diagnosis.

Ghon,[4] Mucha, and Wiesner,[5] among others, reported the finding of gram-negative diplococci in the rectum, vagina, and respiratory tract that were not gonococci, although morphologically they appeared to be such.

An interesting side light on the question of the staining characteristics and morphology of gonococci has been furnished by the work of S. Ramsine and S. Milochevitch.[6] They based their work upon a statement made by Nicolle on the important role played by cocci taking the Gram stain, called Synococci, which are found in postgonococcal urethritis. They quote Delbanco and Lorentz, who studied clinically and bacteriologically diplococci taking the Gram stain. Wichmann and Schlunk confirmed the observation of Delbanco and Lorentz on these diplococci, and showed that they had diminished in virulence. Kandyba succeeded in obtaining a race of diplococci taking the Gram stain, particularly in pure cultures of gonococci in vitro, heated to 40° C. Neither of these others succeeded in transforming this diplococcus into a typical gonococcus.

Several forms of modifications in morphology of gonococci have been described; namely, the macrogonococcus of Le Sodier and J. Verge,[7] elongated forms of A. Cohn,[8] and x forms.[9] Ramsine and Milochevitch, in cultivating gonococci, have found typical forms which appeared and disappeared spontaneously. They observed some that do not take the Gram stain, some that form macrogonococci, others that take the Gram stain. They have changed the appearance of this organism by placing it in contact with gonococcus filtrate, grown in broth or ascitic fluid. They found a remarkable adaptation of this diplococcus in a medium of broth and ascitic fluid. They also found that these organisms varied in their tinctorial properties, and in their size. They proved that they could transform some gram-positive organisms to gram-negative. This was done by reversing the method. Gonococcus S^4 and S^5 returned to the typical form in five generations, Coccus A^1 and A^3 in six generations. By the action of Protargol and by heating at 42° C. for two hours, they changed the gonococcus to a gram-positive diplococcus.

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[1] Stain Technology 2: No. 3, 1927.

[2] Stain Technology 2: 21.

[3] Jour. Bact. 10: 13.

[4] Wien. klin. Wchnschr., 1898; Wien. klin. Wchnschr. 34: 319, 1893.

[5] Handb. d. Geschl. Krkh., Wien u. Leipzig 1911.

[6] Compt. rend., Hebdomadaires des Séances et Mémoires de la Societé de Biologie et des ses Filiales; Societé de Biologie de Belgrade, May 26, 1928, p. 1261.

[7] Compt. rend. Soc. de biol. 92: 323, 1925.

[8] Handb. der Path. Microorg., 1927.

[9] Medycyna doswiadczalna i. spoleczna 1: 359, 1924.

Their conclusions were that the gonococcus is susceptible to pathogenic degradation as well as to evolution in morphology and biology; that specific heat and chemical agents create conditions favorable to this evolutionary behavior; and that this evolutionary process is reversible; that is, the gonococcus may be transformed into gram-positive diplococcus and vice versa.

The author has, for a long time, entertained serious doubt concerning the correctness of diagnoses made at times by gynecologists and urologists who relied on clinical symptoms and history, plus the finding of gram-negative intracellular diplococci in smears from various portions of the urogenital tract; namely, urethra, vagina, Bartholin glands, and cervix. This question has not been given close study and proper consideration even at the hands of laboratory workers. The clinical worker, while dissatisfied with the situation, manifestly could do little or nothing to clarify it. We have for years followed the examples of others in reporting on smears in this stereotyped fashion, "gram-negative diplococci resembling gonococci." In some cases, it is a matter of little or no importance to the referring physician to go any further than this, yet in other cases, particularly of a forensic nature, it is of paramount importance to state whether, from the laboratory standpoint, it *is* or *is not* a gonorrheal infection. Hence some other more exact method of clinching the diagnosis must be used. Culture methods alone seem to be adequate.

CHARACTERISTICS OF NEISSERIA GONORRHOEAE

Morphology: Spheres, 0.6 to 1.0 micron, with one side slightly flattened, particularly when in juxtaposition with another organism. They occur singly, in pairs, or in isolated clumps, but never form chains. Smears from colonies show autolysis, the autolysis becoming more marked the older the colony.

Stain: Gram-negative. They decolorize slowly.

Colonies: Small, translucent, finely granular with lobate margin, grayish white with a pearly opalescence by transmitted light.

Colonies are usually distinct, but may be confluent, particularly if transplanted frequently. Growth may be abundant if a rich medium is used.

Gonococci are characterized by the following reactions:

(a) **Growth only on enriched media**, that is, plates containing serum, ascitic fluid, blood, or supplementary substances.

(b) **Positive oxidase reaction.**—This is carried out according to the method described under R-18. It must be noted that meningococci and many bacilli also give this reaction.

(e) **Fermentation of dextrose, no action on maltose.**—These tests are carried out by inoculating serum broth containing the carbohydrates formula M-7 or cystine trypticase base M-6 with the addition of a suitable indicator and the fermentable substance.

The number of culture media for growing gonococci recommended in the literature is legion, and the results apparently are easy of accomplishment. The desiderata for a workable culture medium for isolation and identification of the gonococcus from female patients are: it should grow the organism quickly; distinctive colonies of gonococci must appear; there must be distinctiveness to the colonies of those other gram-negative organisms that so closely resemble it.

We have elaborated a technic for the cultivation of gonococci which has been eminently successful both in our hands and in those of other workers. It seems to eliminate the objectionable features heretofore encountered. This method published by us in 1926[1] is a modification of Mulzow.[2] The method has been further modified and fully described.[3]

The method embodies the use of a special agar M-37. The plate method is used for all diagnostic purposes. Ascitic fluid is the enrichment fluid in the medium but blood serum from a guinea pig or rabbit may be substituted. Caution must be exercised in obtaining ascitic fluid in a sterile manner since it cannot be heated or filtered to rid it of bacteria. We prefer small Petri dishes for economic purposes; namely, those 75 mm. in diameter. The ascitic fluid and the special gonococcus agar, of course, are kept separate until ready for use. In order to use this material, we place 2 or 3 c.c. of sterile ascitic fluid in a sterile Petri dish. A tube containing the special Gradwohl gonococcus agar is melted and, while fluid, but not hot, with sterile precautions, the agar is poured on the ascitic fluid in the plate, the plate is covered, rotated to mix, and then is allowed to solidify. There should be enough of the combined ascitic fluid and agar when solidified to completely cover the plate to a height of one-half the total height of the Petri dish.

Inoculation of Plates with Gonorrheal Material

Material should be obtained from high up in the organ examined, and should be procured with a platinum loop instead of with a sterile swab. This platinum loop should be a very small one so that only a very small quantity of pus is picked up. The purpose of the very small drop is to get a very thin streak over the surface instead of a heavy streak. In other words, we wish to get distinct isolated colonies. Inoculations are made by streaking the surface of the plate with a small drop on the loop in a zigzag manner so that there will be a definite isolation of the growing bacteria. In making the streak, great care should be exercised that only the very top surface of the medium is streaked; in other words, we should not dig into the medium. Incubate these plates with the covers on top instead of upside down so that there will be an ample supply of water of condensation. With this precaution, together with a pan of water in the incubator, there is no further need for furnishing moisture. Emphasis is to be laid on the fact that in order to grow gonococci, these plates should be inoculated *at once* with material fresh from the living human body.

Plates that have been kept in a refrigerator ready for use should be warmed to body temperature before inoculation. Plates that are dried out should not be used.

Characteristics of the Gonococcus on the Gradwohl Medium

All colonies exhibiting a yellow zone around them are to be regarded as nongonorrheal colonies since gonococci do not ferment levulose, and therefore do not cause a change of color of the indicator (brom cresol purple). Examinations for identification of gonococcus colonies should be microscopic, followed by a stained smear from the colony. In examining the stained smear, look especially for autolysis, which becomes more marked with the age of the colony.

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[1] J. A. M. A. 87: 242, 1926.

[2] J. Infect. Dis. 36: 419, 1925.

[3] Gradwohl, R. B. H.: Urol. & Cutan. Rev. 35: 7, 1931.

If transplants are intended, they should be made within forty-eight hours after the original inoculation, otherwise autolysis will prohibit a successful subculture. See page 1640 for complete description of colonies.

This method has been very useful in practice, both in the male and the female, but its application is possibly more important in the female. This is especially true of cases with forensic features. The method is equally important in diagnosis and in the estimation of cure. In this connection, note the following statement of Vonderlehr,[1] in his review (1941) of the status of the venereal disease control program in mobilization and national defense, "The need continues to exist for the development of facilities for the culture of the gonococcus in public laboratories. The culture remains the most efficient method for the determination of cure in this disease." In the ordinary case of purulent discharge from the male urethra, a demonstration of gram-negative diplococci in the smear suffices in most instances to satisfy a physician that he is dealing with a case of gonococci. But, if there should be any doubt expressed by the patient as to the correctness of this diagnosis, it is the physician's duty to prove that gonococcic infection really exists. This can be done only by culture methods. From time to time, we have seen cases of unspecific purulent urethritis in the male due to organisms other than the gonococcus; it is this type of case that requires a culture diagnosis.

In the female, the question of divorce often hinges upon the result of the diagnosis of gonorrhea. Manifestly, no physician would want to endanger the peace and security of a household by an incorrect diagnosis of gonorrheal infection. In the case of women accused of prostitution and supposed gonorrheal vaginitis, it is equally necessary for the forensic physician to be sure of his ground. Gram-negative diplococci in the male or female are not necessarily gonococci.

It is therefore to be accepted as a proved fact that the growth of the gonorrheal colony on this medium is sufficient proof of gonorrheal infection. On the other hand, the failure to find gonococcic colonies is negative proof that gonorrhea does not exist. The question has been raised in court whether the failure to find gonococci on the plate may not mean the absence of gonorrheal infection. To this argument the answer may be given that a repetition of the attempt and a failure to find gonococci after several examinations on this medium should be sufficient proof to exclude the existence of gonococcus infection in the material examined.

There is another field in which this method is very useful; namely, in the diagnosis of so-called cases of gonorrheal vaginitis in children. Most of these cases show a purulent vaginitis and the presence of gram-negative diplococci in the smears. Some years ago, through the courtesy of Dr. John R. Vaughan, a gynecologist in St. Louis, we were furnished with a number of plates from supposed cases of gonorrheal vaginitis in babies. In twenty-five cases submitted by Vaughan, only two showed positive *Neisseria gonorrhoeae* colonies. The remainder showed gram-negative streptococci. In twenty-three, therefore, out of twenty-five cases, the term "gonorrheal vaginitis" was incorrect.

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[1] Vonderlehr, R. A.: Am. J. Pub. Health 31: 1027, 1941.

Brown, Soule, and Kleine[1] reported the successful use of this medium in cultivating gonococci in cases of true gonorrheal vaginitis in children. Their cultivation work, which was in connection with the treatment of gonorrheal vaginitis in children, showed gonococci in many of the cases examined. The sources of infection in their group were as follows: contact with a member of the family already infected by sleeping with that person, use of common towels, toilet, linens, bath, etc., accounted for most of the cases. They state that only one of the patients developed the infection in an institution. Their cases showed a vaginal discharge of a heavy green-yellow pus, occasionally blood-tinged due to erosions and ulcerations of the mucous membrane. The vulva, hymen, and vagina were usually reddened and often edematous. Their diagnosis was confirmed by demonstration of typical biscuit-shaped intracellular gram-negative diplococci on direct smear, and culture of the organisms on Gradwohl's special hormone agar medium.

Primary Isolation

For primary isolation, culture in a carbon dioxide atmosphere is essential (see pages 1472 and 1575). Gradwohl medium (M-37) and chocolate agar (M-31 to M-33) are the media of choice. Of the latter, chocolate hemoglobin agar with yeast (M-33) is recommended. Modifications of this, M-35 and M-36, are good.

The plates must be incubated for 48 hours, then an oxidase test (R-18) is performed.

Of the preserving media, that of Hirschberg (M-34) is good. Shipping on slants (M-35) is recommended.

Serology

"Typing" of gonococci with the aid of chicken sera is possible but of little practical value. Serotype I is usually from recent cases; II is often an old culture.

ANIMALS

No animal inoculation tests are used.

Neisseria Meningitidis (Neisseria Intracellularis) (Meningococcus)

Morphology: Morphologically similar to gonococcus. They do not autolyze as rapidly as the gonococci.

Stain: Gram-negative.

Colonies: On blood agar, they are small, slightly convex, transparent, glistening. On Gradwohl gonococcus medium, they resemble the macroscopic colonies of the gonococcus.

They grow also on other media used for gonococci, and well under aerobic conditions.

Colonies differ from those of the gonococcus in that they are larger and have a tendency toward confluence.

They cause epidemic meningitis.

The following reactions are characteristic for meningococci:

(a) **Positive oxidase reaction.**—This is performed according to R-18. Hemophilus, Enterobacteriaceae, and a number of other organisms give the same test.

(b) **Fermentation of dextrose and maltose.**—This reaction is tested by inoculating serum broth containing these carbohydrates, M-7, or cystine trypticase base, M-6, with a suitable indicator and 1% carbohydrate of the type to be tested. Meningococci ferment both carbohydrates.

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[1] J. Missouri M. A. 31: 313, 1934.

To isolate the organisms from blood, spinal fluid, petechiae, etc., culture in chopped meat medium M-12, trypticase soy broth M-3, brain heart infusion M-4, or any other semifluid medium containing no inhibitory substances is recommended.

For transportation, use media M-34 and M-35.

SEROLOGY

The Agglutination Test for the Identification of the Neisseria Meningitidis (Meningococcus)

Meningococcus agglutinating serum may be purchased from any reliable biological house. The exact dilution and technic of the test depend in a large measure on the type of serum that is used, and the biological house that manufactures it.

Make the following dilutions of the immune serum in physiologic saline:

1. 1:10, made by adding 1.0 c.c. serum to 9.0 c.c. saline.
2. 1:20, made by adding 0.5 c.c. serum to 9.5 c.c. saline.
3. 1:50, made by adding 0.2 c.c. serum to 9.8 c.c. saline.
4. 1:100, made by adding 0.1 c.c. serum to 9.9 c.c. saline.
5. 1:10, made by adding 1.0 c.c. serum to 9.0 c.c. saline.

Use a 0.2 c.c. pipette graduated in 0.001 c.c. for measuring the serum. (This is the pipette used in the Kahn precipitation test for syphilis for measuring the antigen dilutions.)

Place 1 c.c. of each of the above dilutions of serum into its respective test tube 1, 2, 3, 4, and 5.

Emulsify colonies of the organism to be tested in each of the serums in tubes 1, 2, 3, and 4. Use enough bacteria to produce a slight cloudiness.

Do not place any bacteria in tube 5, as it is the serum control tube.

Place the tubes in a water-bath at 37° C. for several hours, reading at the end of every hour.

If flocculence occurs, the reaction is positive, and the organisms are meningococci.

If no flocculence occurs, the organisms are not meningococci.

Caution: Take great care that the emulsion of the organisms in the serum is complete, or false flocculence due to faulty technic will be observed.

Before placing the tubes in the water-bath, observe for any coarse particles of improperly emulsified colonies.

Typing

Types I and III meningococci contain the same specific carbohydrates; they are therefore classified together as Group I. Type II is the only recognized member of Group II. Types II "alpha" and IV have been found more often in recent years. Type I is found more often during epidemics, Type III between outbreaks.

(a) **Preparation of the Sera, Phair and Noble.—**

Grow "smooth" strains on agar with 5% rabbit blood M-28, M-29. Incubate for 24 hours. Suspend the growth in buffered 0.85% sodium chloride, pH 6.8, and standardize to match number 8 tube of the McFarland nephelometer (page 1477). Inject the suspension into the wing vein of chickens in doses of 0.1 to 0.5 c.c., every second day. A good serum is usually obtained in 10 to 14 days.

To immunize rabbits, use 3 to 5 courses each consisting of 3 daily injections, with one week's intermission between the courses.

(b) **Agglutination Test to Determine Type.—**

The rapid method is recommended.

To 0.1 c.c. of serum add 0.1 c.c. of the bacterial suspension. Prepare two such mixtures with each type serum.

Shake tubes slowly for 2 minutes.

To one tube add 0.85% saline pH 6.8; to the other add 0.85% saline pH 7.8.

Read the agglutinations.

If the meningococci show spontaneous agglutination, the results are better in the tubes containing saline of pH 7.8. Many strains are, however, inagglutinable at this reaction, and react better at pH 6.8. If serial dilutions are tested, dilute the sera 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32. In such cases, agitate the tubes longer; 3 minutes is the recommended time.

Nomenclature of Meningococci*

Common Use	*Recommended by Committee*
I	A
II	B
II alpha	C
IV	D

Quellung Reaction

Type II "alpha" is easily identified by the capsular swelling method similar to that used for pneumococci. Other types do not always have capsules.

Halo Formation

To infusion nutrient agar M-15 add 5% specific meningococcus serum, and pour in plates. Prepare such plates with each type specific serum.

Collect the growth of meningococci from blood agar slants and put large loopfuls on the plates. One may test 6 to 12 cultures on each plate.

Incubate for 48 to 72 hours and inspect for precipitate forming a halo around the colony masses.

WARNING.—If the patient is being treated with sulfa drugs, gonococci and meningococci become atypical. They occur extracellularly, lose their typical shape, and may even become gram-positive.

Neisseria Catarrhalis
(Micrococcus Catarrhalis)

Morphology: Morphology similar to that of the gonococcus. They do not autolyze.

Stain: Gram-negative.

Colonies: They grow on any medium abundantly. They are small, circular, grayish white to dirty white, with erose margin. The twenty-four-hour colony on Gradwohl gonococcus medium resembles the gonococcus twenty-four-hour colony, but does not show striations. They do not ferment any carbohydrates.

Usually nonpathogenic. They may cause severe conjunctivitis.

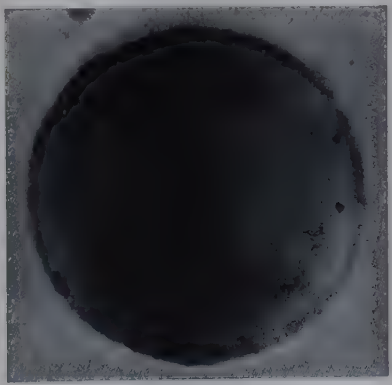

Fig. 357.—Forty-eight-hour colony of *Neisseria catarrhalis* on Gradwohl agar M-37.

Neisseria Sicca
(Micrococcus Pharyngis)

Morphology: Morphology similar to that of the gonococcus. They do not autolyze.

Stain: Gram-negative.

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*From Branham: Bact. Rev. 17: 175, 1953.

Colonies: They grow best on blood agar, or any medium with enriching fluid. They are grayish, somewhat dry, crumbling when an effort is made to remove them. They form acid in dextrose, maltose, levulose, and sucrose.

They do not form a pigment.

Usually nonpathogenic.

Neisseria Perflava

(Chromogenic Group I.)

Morphology: Similar to Sicca.

Stain: Gram-negative.

Colonies: Growth is abundant. Colonies are small, circular, slightly raised, greenish gray by reflected light, greenish yellow and semiopaque by transmitted light. Surface smooth and glistening. Growth adherent to the medium. They form acid in dextrose, maltose, levulose, and sucrose. They are nonpathogenic.

Neisseria Flava

(Chromogenic Group II.)

Morphology and **staining characteristics** similar to *Neisseria perflava.*

Colonies similar to *N. perflava.* They ferment dextrose, maltose, and levulose. Nonpathogenic.

Neisseria Subflava

(Chromogenic Group III.)

Morphology and **staining characteristics** similar to *N. perflava.*

Colonies: Similar to *N. perflava.* They form acid in dextrose and maltose. Nonpathogenic.

GAFFKYA

Gaffkya Tetragena

(Micrococcus Tetragenus)

Morphology: Spheres, usually about 1 micron in diameter, occurring in groups of four, and surrounded by a wide pseudocapsule.

Stain: Gram-positive.

Colonies: Growth is abundant on simple agar. They form acid in dextrose and lactose. Colonies are circular, white, smooth, glistening, entire. Found in sputum in tuberculosis. Pathogenic when injected into guinea pigs.

Klebsiella Pneumoniae

(Friedländer Bacillus)

Morphology: Short, plump rods, with rounded ends, often four to five times as long as they are broad. They occur singly, in pairs, and in thread formation. A wide capsule surrounds the single organism, the pair, the chain or the thread. Nonmotile. Nonspore-forming.

Stain: Gram-negative.

Colonies: Growth abundant, colonies large, mucoid, spreading, white, convex, glistening, entire. Most strains form acid and gas from dextrose and sucrose.

Many strains ferment lactose with acid and gas formation.

May cause fatal lobar pneumonia; bronchitis, bronchopneumonia, etc. They sometimes cause food infections.

This organism is now being classified with Enterobacteriaceae as Aerobacter. It gives a positive Voges-Proskauer reaction and seldom hydrolyzes gelatin. The citrate test is usually positive; indol is not formed. Dextrose is always fermented with gas production; lactose fermentation is the rule.

Klebsiella pneumoniae grows well on ordinary media and may be isolated on agar. The most favorable medium for its differentiation is eosin methylene blue agar M-71 or M-72 on which colonies resembling Aerobacter are usually formed.

Further differentiation is carried out as with other Enterobacteriaceae (see page 1528).

Serology.—Types A, B, C, and X may be differentiated by the capsular swelling method used for the typing of pneumococci, using appropriate sera.

Recently Klebsiella and Aerobacter are considered one group, belonging to more than 100 serologic types.

MYCOBACTERIUM TUBERCULOSIS

Mycobacterium Tuberculosis (hominis) (Koch)

(Bacillus Tuberculosis, Human Type)

Morphology: Rods, from 0.15 to 0.35 micron by 0.5 to 4.0 microns. They occur singly and in occasional threads. May show branching.

Usually occur in isolated clumps of organisms lying side by side.

Stain: Gram-positive and acid-fast. They may show beaded forms which take the dye more brightly than the rest of the organism.

Colonies: Growth is abundant after about six to eight weeks' incubation.

Colonies are dry, crumpled, raised, yellowish, occasionally reddish.

They emulsify with difficulty. Require special media. Cause human tuberculosis; transmissible to guinea pigs.

Mycobacterium Tuberculosis (bovis)

(Bacillus Tuberculosis, Bovine)

Morphology: Rods, shorter and plumper than the human type. From 1 to 1.5 microns in length. Very short forms frequently intermixed with larger forms.

Stain: Irregularly stained. Gram-positive. Acid-fast.

Colonies: On egg medium, they are small, flat, grayish, confluent, moist, glistening. They are the cause of bovine tuberculosis, transmissible to man.

Media favorable for the growth of tubercle bacilli are those of Petrik M-58, Tarshis, M-58a, Whalen, M-58b, Dorset, M-59, Petragnani M-61, Loewenstein M-60, Dubos M-62, and others. Bovine organisms grow better on media without glycerol. Uncontaminated material can be inoculated on media which do not contain inhibitory dyes (brilliant green or malachite green, crystal violet, etc.). Contaminated material must be prepared by concentration methods (see page 1613). Examination of urine and gastric material requires special methods (see pages 1628 and 1646).

Tubercle bacilli are differentiated from smegma bacilli by the use of the Pappenheim stain (page 1412); from leprosy bacilli by their growth on egg media.

Refer to page 1475 for culture of tubercle bacilli by the egg culture method.

SEROLOGY*

Tuberculin Skin Tests of Von Pirquet for the Diagnosis of Tuberculosis

The Scratch Test

Koch's old tuberculin (O. T.) may be obtained from any reliable biological supply house.

Cleanse the patient's forearm with alcohol.

Make three scratches (superficial) in the skin, each scratch about ½ inch in length, and at a distance of about ½ inch from each other.

Into the first two scratches (test), rub a small amount of tuberculin, using a sterile match stick.

Into the third scratch, rub a small amount of sterile physiologic salt solution (control).

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*See page 2092 for the Weltmann reaction.

Allow these to dry.

Instruct the patient to return to the laboratory for examination at the end of 24, 48, and 72 hours.

If the reaction takes place within twenty-four hours, he need not repeat the visits.

A positive reaction is characterized by a slight swelling along the scratch, and an area of inflammation surrounding the inoculation.

The saline control should show no swelling or redness. If there is a reaction due to saline, the result of the test is unreliable.

A negative reaction shows no change in the skin.

A positive reaction persists for several days.

The Koch Subcutaneous Tuberculin Test

In this method Koch's old tuberculin is used. This is a filtrate of a concentrated bouillon culture of tubercle bacilli containing the toxin of the bacteria. It produces a focal as well as a general reaction. Before applying the tuberculin test take the temperature of the patient for several days to determine variations. Contraindications to the test are a markedly febrile course, general miliary tuberculosis, acute or advanced tuberculosis, tuberculous meningitis, the terminal stages of other diseases, and during convalescence from acute febrile diseases. The usual dose is 0.5 milligram.

A positive reaction consists in an elevation of at least one degree Fahrenheit, focal reaction, and a local reaction at the site of the injection. If no reaction is produced, repeat the injections, increasing the dosage gradually every three to five days until as much as 5 milligrams have been given.

Intradermal Tuberculin Test of Mantoux

In this test a measured amount of old tuberculin is injected between the layers of the skin. A positive reaction indicates a case of present tuberculosis infection. A negative reaction is of the greater value in diagnosis. For this test we need the following solutions: five solutions of old tuberculin: 1 in 10; 1 in 100; 1 in 1000; 1 in 10,000; 1 in 20,000. They may be purchased ready for use, and if kept in a cool place, will remain potent for three years.

Technic.—

Sterilize a 1 c.c. tuberculin syringe and several 26 gauge ½ inch hypodermic needles. Sponge the forearm with alcohol. Fit a needle to the tuberculin syringe, with the bevel of the needle on the same side as the graduations on the syringe. Fill the syringe to 0.2 with the old tuberculin. Pinch the skin and press in the needle until the lumen just disappears. The injection raises a white wheal. In patients who have had recent hemoptysis, skin or joint disease, the initial dosage should be 0.1 c.c. of 1 in 10,000.

Read the reaction on the second to fourth day. An area of erythema ⅜ inch or more in diameter is positive. The more marked reactions show swelling and at times necrosis. When the reaction is negative repeat the test, using 0.1 c.c. of 1:1,000 dilution, and if still negative forty-eight hours later, repeat, using 0.1 c.c. of a 1:100 dilution, etc.

The Vollmer Tuberculin Patch Test*

This is a quick, accurate, painless test obviating the use of instruments as well as injury to the skin. Thoroughly cleanse and defat, with acetone, the area of the skin of the inner side of the forearm or sternum (recommended for children) or upper spine (recommended for infants). Then apply the patch firmly. Instruct the patient not to disturb the patch for 48 hours, and to avoid getting it wet. The second day after removal, the patient returns to the laboratory for interpretation of the test.

A *positive* reaction appears as an infiltrated elevated reddened square. The central control square appears pale. If the patient is sensitive to adhesive tape, there may be irritation due to the tape, but this does not interfere with reading of the reaction.

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*Material for the Vollmer Patch Test may be obtained from Lederle Laboratories, Inc., New York, N. Y.

If directions for making and reading the test are carefully carried out, the results will be found superior to the von Pirquet test and comparable to those of the Mantoux test. Late reactions should be reported to the physician.

Interpretation of Tuberculin Reactions

The tuberculin reaction is a reliable test for hypersensitiveness to tubercle bacillus toxin; by that we mean past or present tuberculous infection. The negative reaction is of most value in clinical diagnosis. When tuberculosis nears a fatal termination, sensitiveness to the tuberculin injection is depressed so that we have no reaction in any case of tuberculosis just before death.

In children, a negative reaction is useful to exclude tuberculosis in chronic cervical adenitis, prolonged anorexia, debility following bronchopneumonia, whooping cough, or measles. A positive reaction in a child of two years or over does not of itself prove an active tuberculosis. It is well to use additional data to establish diagnosis. If complete healing of a tuberculous infection occurs and the bacilli die, the tuberculin reaction after a time becomes negative. Sometimes, infection becomes well walled off, often in a calcified nodule, but still the bacteria remain alive and we note a positive tuberculin reaction. A markedly positive Mantoux reaction in a patient with indefinite physical signs suggests some active focal infection which, under unfavorable hygienic conditions, may spread and form extensive disease.

See the chapter on Hematology for changes in the blood picture upon injections of tuberculin, method of Bredeck, page 795.

ANIMAL INOCULATION

Animal Inoculation.—Test guinea pigs for sensitivity to tuberculin by injecting 1 mg. of old tuberculin intracutaneously. Wait three days, and if the test is negative, inject the material to be examined for tubercle bacilli into the groin, under the skin. It is advisable always to inject guinea pigs in the same side, for example, the left side.

After 4 weeks, make tuberculin skin test on the animal. If positive, report as such.

Kill animal after eight weeks and perform an autopsy, including histologic examination of regional glands and organs.

Mycobacterium Leprae

(Leprosy Bacillus) (Hansen Bacillus)

Morphology: Rods, 0.3 to 0.5 by 1 to 8 microns, parallel sides and rounded ends, often in clumps or rounded masses, or in groups, side by side, rarely bent or curved.

Stain: Gram-positive, staining unevenly, at times beaded; acid-fast.

Colonies: This organism has never been successfully cultivated. It is considered the cause of leprosy, or Hansen's disease.

The organisms stain red with the Ziehl-Neelsen stain, resembling the tubercle bacillus. They differ from the tubercle bacillus, being usually grouped in masses held together by mucus, called ''globi,'' or in cigarlike bundles. They are found in most cases of leprosy in the mucous membranes of the nose, in the lymph which oozes from superficial erosions of the skin, and in the scrapings from the corium or after curetting of the epidermis. They are usually also found in huge numbers in tissue sections in the lepromatous types. The bacilli are also found in the peripheral nerves, as well as in the liver and other viscera in advanced cases of Hansen's disease.

It is not known whether the bacilli live outisde human tissues. The actual mode of contagion is also obscure. Some believe that prolonged personal contact with the patient must exist before the disease is acquired. Direct inoculation on the skin with development of an initial lesion has been reported by some observers, but it is generally accepted that the disease may have access to man through the mucous membranes of the nose and mouth.

The Histamine Test

Place a drop of 1:1,000 solution of histamine phosphate on the affected skin to be tested.

Place another drop at a point between the suspected area and the adjacent normal skin, and a third drop on the normal skin.

Lightly prick the epidermis through these drops, using the point of a hypodermic needle, being careful not to cause bleeding.

Immediately dry the drops of histamine with cotton, and carefully watch the sites of the test.

Within 25 to 40 seconds, the *normal skin* shows an area of erythema with irregular outline, usually 5 to 20 mm. in diameter, later reaching as much as 5 cm.

In 60 to 100 seconds, a wheal forms in the erythematous area, centered by the point where the needle entered the epidermis. After 3 minutes, a hemorrhagic point may appear at the site of the needle prick. The wheal usually measures 2 to 5 mm. in diameter.

The reaction persists for approximately 20 minutes, but at times the erythema and the wheal are still present after 45 minutes. There are individual variations due to age, room temperature, and local circulatory conditions. The test cannot be made on the skin of Negroes.

Between the affected and the normal skin, the test shows the same succession of phenomena on the normal half of the skin, but on the affected half, the erythematous reaction stops abruptly and is absent on that part of the skin, but the wheal is always present without the peripheral erythema.

On the *anesthetic skin,* erythema is wholly lacking, while the wheal is present and remains the usual length of time.

The test causes no subjective sensations, either on the normal or on the affected skin.

The test is read as normal or abnormal, and not as positive or negative.

Any type of degenerative peripheral neuritis may give an abnormal histamine test, and therefore it is not exclusively diagnostic of leprosy. The test is not reliable when performed at a room temperature below 24° C. or when performed on aged persons with slow or impaired peripheral circulation. It is important in the differential diagnosis between leprosy and syringomyelia, since in the latter condition the histamine test is normal.

The Lepromin Test (Mitsuda Test)

This test consists essentially of an intradermal injection of lepromin, an antigen prepared from tissues rich in Hansen bacilli. It is made by boiling lepromatous tissues in isotonic solution of sodium chloride for 1 hour, after which the mass is ground in a mortar, and 20 c.c. of 0.9% solution of sodium chloride are added for each gram of ground tissue. After repeated and thorough mixing and grinding, the supernatant fluid is pipetted off, filtered through gauze, and stored in a sterile container.[1]

The liquid is autoclaved at 120.° C. for 15 minutes, 0.5% phenol added, and distributed to rubber-capped vials ready for use. It is a cloudy, milky emulsion containing numerous bacilli (globi), and the tissue elements of the leproma. If kept in a dark, cool place, it retains its activity for a long time Seitz-filtered antigens are inactive.

Technic.—

Inject 0.1 to 0.15 c.c. of lepromin intradermally either into the skin of the scapular region or upon the anterior surface of the thigh.

At the end of 24 to 48 hours, there is a light erythema which is attributed to the presence of foreign protein material and which disappears in a few days, usually in 3 or 4. The site of the test then remains apparently normal until the beginning of the third week when, if the test is positive, a slight papular infiltration is noted, gradually increasing in size until it attains the size of a pea. The infiltration is deep and its color is bright red. There might be some tenderness on pressure and, occasionally, formation of a central necrotic focus with ulceration, resulting in a permanent scar.

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[1] Rotberg: Rev. brasil. leprol. 5: 45, 1937.

The test should be evaluated on about the thirtieth day after the injection. If there is no reaction or only a small (less than 5 mm.) infiltration, the test is negative.

The test is positive in about 90 per cent of normal individuals, in practically all cases of tuberculoid leprosy, and in about 50 per cent of patients with the simple inflammatory type. It is negative in practically every case of lepromatous leprosy, in about 10 per cent of apparently normal persons, and in about 50 per cent of patients with simple inflammatory leprosy.

It is a *prognostic test.* If the test is negative in a person without symptoms of leprosy, that person is susceptible to the disease. The test is applied to prospective employees of leprosaria; those with negative Mitsuda tests are not permitted to serve in such institutions.

Diagnosis

Diagnosis is made by smears from the mucous membrane and by making sections of suspected lesions. These lesions show granuloma with typical bacilli present. The bacilli multiply in the lymphatics of the corium and subcutaneous tissues, with development of large monocytic cells which engulf the organisms in such large numbers that a microscopic view of such a cell shows it filled with round, acid-fast bacilli. These cells have been called "leprosy cells."

The complement fixation test for syphilis is frequently positive and can be of no aid in the differential diagnosis. This might be due also to a concomitant infection with syphilis or yaws.

Table 144[2] shows the correlation of the clinical manifestations of leprosy with the histopathologic changes, the immunologic response, and the bacteriologic findings.

TABLE 144[2]

CLINICAL	PATHOLOGY	IMMUNOLOGY	BACTERIOLOGY
Lepromatous Nodules, lepromas, diffuse infiltration "en nappe," loss of eyebrows, rhinitis, leonine facies, fever, and peripheral pains Mutilations: Nose, extremities	Diffuse infiltration of dermis, foam cells, "Leprazellen" with globi. Atrophy of the epidermis Atrophy of hair follicles and glands Areas of necrosis	Mitsuda test negative in 90%	Skin: Many bacilli, singly, in bundles, and globi; in sections and capillary blood Nerves: Numerous bacilli singly and in bundles in and between nerve fibers in sections
Tuberculoid Flat, dark red infiltrations, well-defined geographic, annular borders; enlargement of nerves, no fever, atrophy of hands. Includes the old neural types	Patchy sarcoidlike areas of infiltration around blood vessels, hair follicles, and glands. Foam and giant cells, no globi Nerves: Profuse infiltration, same type	Mitsuda test positive in 90%	Skin: Occasional bacilli in sections Nerves: Occasional bacilli in sections
Simple Inflammatory Macular lesions with little or no elevation; pink, red, or dyschromic areas of anesthesia without clinical changes	Lymphocytic infiltration around blood vessels, glands, and hair follicles. No foam or giant cells, no globi	Mitsuda test positive in 40 to 50%; negative in 50 to 60%	No bacilli or only occasional in sections

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[2] V. Pardo Castelló in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, St. Louis, 1951, The C. V. Mosby Co.

Mycobacterium Lacticola
(Smegma Bacillus)

Morphology: Rods, usually more plump than the tubercle bacillus, variable in size.

Stain: Gram-positive and acid-fast.

Colonies: On glycerin agar, isolated colonies, becoming lobulated and velvety, grayish, butyrous. Differentiated from tubercle bacillus by its inability to produce lesions in guinea pigs. Nonpathogenic.

Another acid-fast nonpathogen is the *Mycobacterium phlei* (the hay bacillus), which is probably identical with the smegma bacillus.

CORYNEBACTERIA

Corynebacterium Diphtheriae
(Bacillus Diphtheriae)

Morphology: Rods, varying greatly in dimensions, 0.3 to 0.8 micron in breadth and 1.0 to 8.0 microns in length. They occur singly, or in palisade formation. They are straight or slightly curved, frequently swollen at one or both ends. They do not stain uniformly, but have granules or bars of more deeply staining properties.

Stain: Gram-positive.

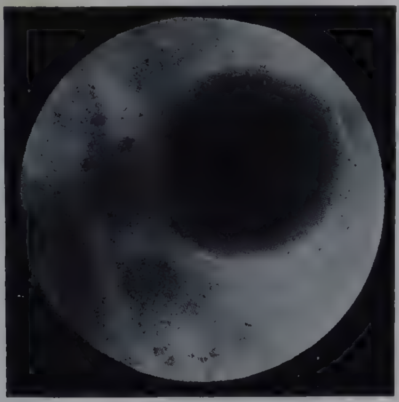

Fig. 358.

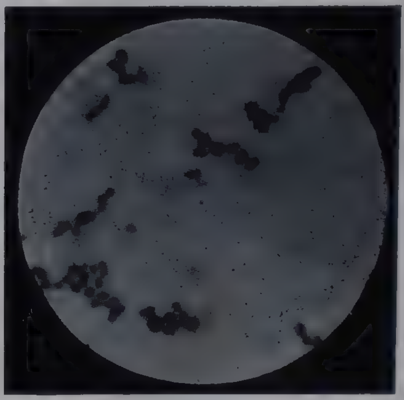

Fig. 359.

Fig. 358.—Colony of *Corynebacterium diphtheriae* on blood agar.
Fig. 359.—*Corynebacterium acnes.* (×950)

Colonies: On blood serum media they are grayish to creamy, moist, smooth, slightly raised, margin entire. On blood agar, they turn the blood brown.

Growth is moderately abundant. They are the cause of diphtheria. They kill guinea pigs within four days.

All strains ferment dextrose but not lactose or sucrose. The best differentiation of the types is determined by a study of the growth on tellurite media M-41 to M-43.

THE GRAVIS, MITIS, AND INTERMEDIATE TYPES OF C. DIPHTHERIAE

These types of *C. diphtheriae* were first described by Anderson, Happold, McLeod, and Thomson[1] in 1931. They noted a difference in appearance of diphtheria bacilli when grown on heated blood agar medium containing potassium tellurite. These two different colonies, black or gray-black, when subcultured and tested by the usual methods, gave all the reactions of typical toxigenic *C. diphtheriae.* They were differentiated in other ways—by their power to ferment starch and glycogen, by their growth in broth, and by the production of hemolysis. It is interesting to note that in Leeds, where this study was made during a severe epidemic of diphtheria, one of the two types was isolated from the severe and fatal cases, while the other type was isolated only from mild cases. For this reason McLeod and his associates called one type *C. diphtheriae gravis,* and the other *C. diphtheriae mitis.* They noted, too, the existence of a certain number of intermediate strains, corresponding neither to the gravis nor to the mitis type. Subsequently these same workers noted that the "intermediate" strains formed a third type, with well-differentiated characters of its own. This type came from cases which were more severe than those cases caused by mitis strains but less severe than those cases caused by gravis strains. Robinson and Marshall,[2] in a similar study in Manchester, confirmed the occurrence of the three types described by McLeod and his colleagues; but in the Manchester series the intermediate strains were more numerous than either the gravis or the mitis.

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[1] J. Path. Bact. 34: 667, 1931.

The characteristics of these three types may be summarized[3] as follows:

> "**C. diphtheriae gravis.**—Gives a gray or gray-black 'daisy head' colony on blood-tellurite medium, the degree of striation of the surface and crenation of the edge varying considerably. Ferments dextrin, starch, and glycogen (in addition to dextrose and maltose, which are fermented by all types of *C. diphtheriae*). Gives surface growth and granular deposit in broth, generally with an early (2-3 days) reversal of the pH to the alkaline side. Usually nonhemolytic. Stained films from 18-hour cultures on Loeffler's medium (the morphology of *C. diphtheriae* grown on the blood-tellurite medium is quite atypical) show bacillary forms with one or two darkly staining areas, the rest of the cell staining very lightly. Metachromatic granules are rare, and when present are very small. Some 60 per cent of gravis strains conform to this morphology, the others may show much the same appearance as mitis or intermediate strains.
>
> "**C. diphtheriae mitis.**—Gives a convex, smooth, black, shining colony on blood-tellurite medium, with an entire edge. It does not ferment starch or glycogen. The fermentation of dextrin is variable. Gives diffuse turbidity in broth, with a nongranular deposit. Surface growth is infrequent; when it occurs, it is soft. There is later a reversal of pH to the alkaline side (4 to 5 days). It is usually hemolytic. The bacilli stain very irregularly, and metachromatic granules are very prominent.
>
> "**C. diphtheriae (intermediate).**—Gives a small, flat colony on blood-tellurite medium, with a central, raised, black or gray-black portion, and a lighter, slightly crenated periphery. Does not ferment starch or glycogen. Fermentation of dextrin is variable. Gives a fine granular deposit in broth, with no surface growth. There is no reversal of pH. This type is consistently nonhemolytic. The morphology is very characteristic, about 98 per cent of the bacilli being barred."

The most reliable criteria for the differentiation of these three types are the type of colony formed on blood-tellurite medium and the power to ferment starch and glycogen.

The three types of McLeod are also differentiated from one another in antigenic structure. Ewing[4] examined 106 gravis or starch-fermenting and 50 non-starch-fermenting strains. The gravis strains fell into five different serologic types, and none of these were antigenically similar to any of the non-starch-fermenting strains. Thirty-five mitis strains yielded four different antigenic groups, and 15 intermediate strains yielded four groups. Murray[5] examined 78 gravis, 70 mitis, and 102 intermediate strains, and was able to differentiate three different antigenic types of gravis, four of mitis, and four of intermediate. There was no antigenic relationship between the three different types.

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[2] J. Path. Bact. 38: 73, 1934.

[3] Topley, W. W. C., and Wilson, G. S.: Principles of Bacteriology and Immunity, quoted by permission of Wm. Wood & Company, pages 341-342.

[4] J. Path. Bact. 37: 345, 1933.

[5] J. Path. Bact. 41: 439, 1935.

DIFFERENTIATION OF DIPHTHERIA BACILLI FROM OTHER ORGANISMS BY POTASSIUM TELLURITE MEDIUM

Tellurite and copper sulphate are used in media selected for the primary isolation of diphtheria bacilli to inhibit the growth of gram-negative rods and many cocci. Streptococci and staphylococci, however, grow on these media. The action on Loeffler's slant M-10 is based upon the more rapid multiplication of the diphtheria bacillus on these media. When a Loeffler slant is inoculated and not observed for 24 hours or longer, the secondary flora will outgrow the Corynebacteria. Therefore Loeffler's medium should be used only when examinations of the slants after 10, 14, 16, and 18 hours are contemplated.

From Loeffler's medium the growth must be transferred to a tellurite plate (M-41 to M-43), from which are inoculated serum broth with dextrose and sucrose M-7, cystine trypticase (M-6) with dextrose and sucrose or proteose-peptone broth with dextrose and sucrose M-5. These tubes must be observed for 5 days.

Transfer pure cultures to Loeffler or blood agar tubes M-28, M-29, and use the growth to test for virulence.

VIRULENCE TESTING

(a) **Chicken Test of Frobisher and Associates.***—Inject Plymouth Rock or Rhode Island strain chickens, 7 to 20 days old, with 50 to 100 units of antitoxin into the areolar tissue dorsally between the wing insertions.

After one hour inject about 1 to 2 c.c. of the bacterial growth from Loeffler or blood agar tubes into the same area. Use four chicks, two protected with the antitoxin and the others unprotected.

Virulent strains usually kill both unprotected chickens within 24 hours. In surviving animals, wing paralysis may indicate the toxicity of the culture.

(b) **Subcutaneous Injection.**—Inject one guinea pig with 3 units of diphtheria antitoxin per each gram of weight, intraperitoneally, 24 hours before the experiment.

Inject this guinea pig and an unprotected guinea pig with the washings from the surface of a Loeffler slant, making the injections subcutaneously. Observe for 10 days. Death occurs most often within 5 days. Virulent strains produce a circumscribed local in filtrated lesion with necrosis but the control pig shows a normal skin.

(c) **Multiple Intracutaneous Test.**—Several cultures may be tested on the same animals. Inject one guinea pig with 3 units of diphtheria antitoxin per gram weight, intraperitoneally, 24 hours before the experiment.

Inject 0.15 c.c. of the suspensions of growth on Loeffler slants into the shaven skin of the abdomen of a guinea pig protected with antitoxin and into another guinea pig not so protected.

Observe for necrosis in 24 hours and again in 48 and 72 hours.

(d) **Plate Method, Elek[1] and King[2] et al.**—Prepare sterile paper strips; soak them in diphtheria antitoxin, 500 units per c.c.

Press strips into medium M-44.

Streak bacteria from 18-hour-old broth culture in M-3, across the strips.

Observe for zones of precipitation in 24, 48, and 72 hours.

SEROLOGY

The Schick Test for Determination of Susceptibility to Diphtheria Infection[3]

Schick toxin and controls may be purchased from any reliable biological house. This toxin is diluted and ready for use.

0.1 c.c. of this mixture is 1/50 the minimum lethal dose of diphtheria toxin. This is the quantity to be injected.

Disinfect the flexor surface of the forearm.

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*Am. J. Hyg. 35: 381, 1942.

[1] Brit. M. J. 1: 493, 1948.

[2] Am. J. Pub. Health 39: 1314, 1949.

[3] White, B., Bunney, W. E., and Malcolm, W. G.: J. Immunol. 32: 93, 1932.

Withdraw the mixture in the vial using a 1 c.c. tuberculin syringe with a 23 gauge ½ inch needle. The syringe should be graduated in 0.1 c.c. The beveled edge of the needle should be in line with the graduations of the syringe.

Push the needle between the layers of the skin until the point is well covered by the skin.

The injection is to be made intradermally; hence, care must be taken not to give a deep inoculation. The point of the needle can be seen under the skin if the injection is made correctly. Inject 0.1 c.c. of the mixture between the layers of the skin. A small white wheal will form.

Saline Control.—About 2 inches below this test puncture, inject 0.1 c.c. of sterile physiologic saline.

Protein Control.—After the injection of the Schick toxin inject 0.1 c.c. of the heated mixture (control) intradermally about 2 inches below the saline injection. If the reaction is not read until the third or fourth day, this last injection may be omitted. Protein reactions occur within twenty-four hours, and can therefore be eliminated in the final reading.

Reading the Reaction

The patient is instructed to return to the laboratory every day for four days. If a reaction occurs within twenty-four hours, it usually is not a diphtheria reaction. If the reaction is due to a hypersusceptibility to saline, the saline control will show a zone of inflammation, and a swelling. If the reaction is due to proteins, the reaction is much more vigorous than the true Schick reaction, and will reach its height within twenty-four to forty-eight hours.

A negative reaction is indicated by no change in the skin.

A positive reaction is accompanied by a swelling and a zone of inflammation around the point of inoculation. The zone of inflammation is about 1 or 2 centimeters in diameter around the point of inoculation. The inflammation gradually disappears, and is replaced by a browning of the skin accompanied by a slight area of necrosis. The slight brown discoloration may persist for several months.

A positive Schick test is indicative of susceptibility to diphtheria infection.

A negative reaction indicates immunity to diphtheria infection.

After the toxin has been diluted with the saline, it cannot be used for a period longer than twenty-four hours.

Corynebacterium Pseudodiphtheriticum
(Pseudodiphtheria Bacillus of Hoffmann)

Morphology: Rods, with rounded ends, 0.3 to 0.5 by 0.8 to 1.5 microns, fairly uniform in size, without swollen ends. Barred forms may be found, although polar granules are usually seen.

Stain: Gram-positive.

Colonies: On blood serum media, they are opaque, grayish to cream-colored, smooth, homogeneous, entire, fairly abundant. May be differentiated from diphtheria bacilli by their inability to produce disease in lower animals. Nonpathogenic. They do not ferment any carbohydrate.

Corynebacterium Xerose
(Bacillus Xerosis)

Morphology: Rods, showing polar staining, occasionally club-shaped forms.

Stain: Gram-positive.

Colonies: Thin, grayish, adherent. Growth scant, and slow. Nonpathogenic. They form acid in dextrose, levulose, galactose, maltose, and sucrose. Cultivated on Loeffler's blood serum medium or Petroff's egg medium.

Corynebacterium Acnes

(Acne Bacillus)

Morphology: Rods, varying in dimensions, usually 0.5 by 0.5 to 2.6 microns, sometimes slightly club-shaped. Show alternate bands of stained and unstained material. In transplanted cultures, the rods occasionally assume coccoid forms. They group in palisades.

Stain: Gram-positive.

Colonies: Small, grayish colonies, circular, transparent. Original growth on Petroff's egg medium under micro-aerophilic conditions. Original growth scant; transplants may show abundant growth of glistening colonies. They show slight acid formation in dextrose. Found normally in hair follicles and sweat glands; often present in acne.

ENTEROBACTERIACEAE

Gram-negative, nonsporeforming microorganisms, all of which ferment dextrose. Mostly inhabitants of the intestinal canal. Under pathologic conditions, isolated from blood, pus, urine, etc.

Salmonella Typhosa

Morphology: Rods, 0.6 to 0.8 by 1.0 to 1.5 microns, occurring singly, in pairs, short chains or threads. Motile with peritrichous flagella.

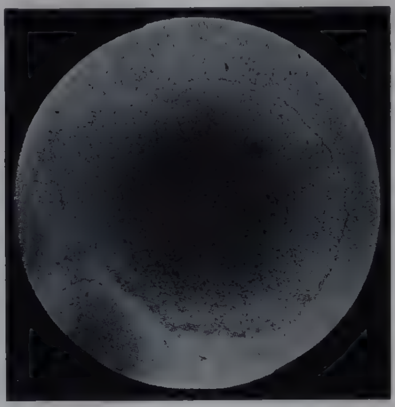

Fig. 360.

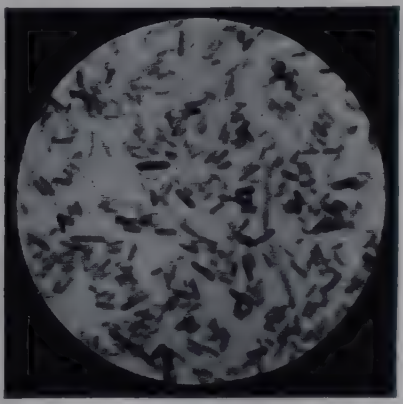

Fig. 361.

Fig. 360.—Colony of *Salmonella typhosa* on nutrient agar. (×950.)
Fig. 361.—*Salmonella typhosa.* (×950.)

Colonies.—Colonies are whitish to grayish, transparent to opaque, often with undulate margins. On media with ferric salts, colonies are more yellow and brownish. On agar media with bismuth sulphite, M-69 and M-70, after 48 hours they are flat, with broad dark halo and metallic sheen in reflected light; flat or convex; grow into the medium.

Biochemistry.—Biochemistry is the same as that of Salmonellae but *S. typhosa* does not form gas. It is a moderate producer of hydrogen sulphide.

SEROLOGY

S. typhosa has the antigenic structure IX, XII:d of Salmonellae. Types may be distinguished by the use of phage-typing introduced by Craig and Yen[1] which is carried out by mixing inocula of the organism tested with type phages on a 1% agar plate. As many as 12 tests may be performed on one plate. The plate is incubated and the action of the phage suspensions upon the growth of the typhoid bacilli read.

Only cultures containing Vi antigen are considered for phage typing. Exceptions, however, were described. The phage typing has great importance for public health investigations.

Salmonella[2]

Salmonellas have become one of the major problems of physicians, veterinarians, epidemiologists, and laboratory workers. Hundreds of types and varieties belonging to this genus are known to date, and the number of newly described strains increases constantly. Attempts to simplify this complex group of microorganisms by considering all or most of the types of *Salmonella* just variants of one bacterium were shattered by the difference in epidemiologic nature of the diverse strains. The wide dispersion of Salmonellas constitutes a health problem of staggering proportions. These organisms are pathogenic for man and animals and they are present in all places inhabited by these hosts, thereby creating problems in all climates.

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[1] Canad. Pub. Health J. 29: 449 and 484, 1938.
[2] Felsenfeld: Am. J. Clin. Path. 12: 584, 1945.

The old theory, called the ''Doctrine of Kiel,'' taught that there are ''human adapted'' and ''animal adapted'' Salmonellas, the first group causing generalized infections, the second attacking man rarely and in such cases producing only gastroenteritis. Thanks to the efforts of Hormaeche and his school, the ''Doctrine of Montevideo'' is now being generally accepted. This theory asserts that persons contracting Salmonella infections react in very different fashions depending on several factors, among which the age and the general health of the patient, and, to a lesser extent, the strain of the organism are most important. Thus, septic infections and meningitis are more frequent in infants and in young animals, due to the lesser resistance of this age group. Similarly, higher mortality is observed in old people, mainly among those suffering from other diseases or confined to institutions.

The clinical picture of salmonellosis may be described as follows:

1. Salmonella fever, the ''paratyphoid disease'' of former authors.
2. Salmonella septicemia, with irregular temperatures, and often grave prognosis, producing eventually local inflammatory lesions.
3. Salmonella gastroenteritis, which, according to Hormaeche and associates may be
   a. Choleriform
   b. Dysentery-like
4. Localized infections, including appendicitis-like syndromes, genitourinary inflammations, cholecystitis, etc.
5. Symptomless carrier status.

Statistics do not always differentiate between Salmonella fever and Salmonella septicemia. These two groups are, therefore, united into one.

While most Salmonellas follow the general rules of the Montevideo doctrine, it is also apparent that the type involved in the infection may play an important role in the disease produced; e.g., ''paratyphoid bacilli'' and *S. sendai* are more prone to produce Salmonella fever, while *S. choleraesuis* often causes septicemia; *S. give* provokes only enteritis; and *S. typhimurium* can be the cause of any form of salmonellosis. With the increase in the number of observations of Salmonella strains in man, however, more and more cases are described which tend to prove that most of these organisms can produce, under favorable circumstances, any form of salmonellosis.

NOMENCLATURE, MORPHOLOGY, AND BIOCHEMISTRY

The generic name, *Salmonella,* is derived from Salmon, the investigator who described the first member of the genus, *S. choleraesuis.* Difficulties in the exact limitation of the species belonging to this genus cause most authors to prefer the taxonomic designations ''types'' and ''members'' instead of "species." A variety of sources was used as origin for the nomenclature employed for these types. In the beginning, organisms which caused typhoid-like fevers were designated as paratyphoid bacilli A, B, and C. Other members of the group were named according to the disease caused: *S. enteritidis, S. typhisuis, S. abortus ovis,* etc. Certain types received names of the animals in which they were first discovered, as in the case of *S. anatis* or *S. meleagridis.* Salmonellas rarely bear the name of the investigator who first described them, as *S. schottmuelleri.* Eventually it became an established custom to name newly discovered types after the state or country where they were isolated: *S. virginia, S. georgia, S. panama.* If a new strain is described in a state the name of which already occurs on the list of these organisms, the name of the town where the organism was found is employed: *S. illinois—S. urbana, S. champaign; S. kentucky—S. lexington.* When the names of the state and the city have been used to designate Salmonella types and a new strain is discovered in the same city, the name of the hospital where the patient is treated serves this purpose, e.g., *S. berlin—S. virchow, S. cophenhagen—S. bispebjerg.* Interesting examples of nomenclature are *S. selandia* and *S. orion,* named for the ships which carried the first patient suffering from such infection.

The genus *Salmonella* is composed of gram-negative, aerobic and facultative anaerobic, nonsporeforming, mostly motile bacteria, which produce acid and gas from dextrose, mannitol, and maltose, but do not ferment lactose, sucrose, salicin, or adonitol; do not produce indol or acetyl methyl carbinol; do not coagulate milk and do not liquefy gelatin. All members of the genus possess antigens which fit into the White-Kauffmann scheme.

Among the organisms in which the taxonomic position in this genus is dubious, the foremost place is occupied by *Salmonella typhosa,* the typhoid bacillus. This organism forms no gas, only acid, from fermentable substances. The antigenic structure and the disease which it causes, however, have assured the typhoid bacillus a permanent place in the Salmonella scheme. Nongas-forming, also called "anaerogenic," variants of Salmonellas, other than *S. typhosa,* are not uncommon in the literature, as strains of *S. typhimurium, S. schottmuelleri, S. enteritidis* which produced acid and no gas. *S. gallinarum,* also called *Shigella gallinarum,* is another organism which has a typical antigenic structure but forms acid with no gas from fermentable substances. As it is nonmotile, it is often classified with Shigellas. Another nonmotile organism is *S. pullorum,* which, in addition to this irregularity, does not always ferment maltose. Hinshaw and associates, however, showed that strains which do not ferment maltose are typical in other respects.

There are numerous exceptions to the definition of the genus *Salmonella,* mainly biochemical in nature. These strains are nevertheless accepted because of their other qualities. For instance most strains of *S. typhisuis* do not ferment mannitol. There are indol forming variants of *S. eastbourne, S. enteritidis,* and *S. panama,* as well as acetyl methyl carbinol producing variants of *S. choleraesuis.* Finally, *S. dar es salaam, S. abortus bovis, S. canastel,* and *S. schleissheim* liquefy gelatin. These differences, however, are rather rarely encountered and there is little doubt that they may be neglected in routine laboratory work.

Many attempts have been made to use biochemical reactions as a basis for the differentiation of Salmonella types. One of them was the desire to divide

Salmonellas into two groups one of them forming, the other not forming hydrogen sulphide. Most of these organisms form hydrogen sulphide. The detection of this reaction, however, depends on the richness of the peptone used in the preparation of the culture media on sulphur compounds and on the indicator employed in the detection. The tabulations of hydrogen sulphide producing Salmonellas used in most laboratories, however, are not compiled for the purpose of detecting traces of such biochemical activity, but reflect experience with many strains on standard media. According to these charts, all strains of *S. paratyphi* (with the exception of its variety durazzo), *S. choleraesuis* (the "american" or diphasic variety), *S. abortus equi, S. typhisuis;* many strains of *S. gallinarum, S. sendai,* and *S. newcastle,* and a few strains of *S. dublin, S. abortus ovis,* and *S. typhosa* do not possess the ability to change the indicator used in Kligler's or in Hajna's media or in the bismuth sulphite agar.

TABLE 145.—NOMENCLATURE OF SALMONELLAS

ACCEPTED DESIGNATION	SYNONYMS
Salmonella paratyphi	*Bacillus paratyphosus* A *Bacterium paratyphosum* A *Salmonella paratyphi* A Paratyphoid A bacillus
Salmonella schottmuelleri	*Bacillus paratyphosus* B *Bacterium paratyphosum* B *Salmonella paratyphi* B Paratyphoid B bacillus
Salmonella typhimurium	*Bacillus pestis cavae* *Bacterium aertrycke* *Salmonella enteritidis* "Breslau" *Salmonella aertrycke* *Aertrycke bacillus* Breslau bacillus
Salmonella hirschfeldi	*Bacillus paratyphosus C* *Bacterium paratyphosum* C *Salmonella paratyphi* C Paratyphoid C bacillus
Salmonella choleraesuis	*Bacillus suipestifer* *Bacterium suipestifer* *Salmonella suipestifer* Hog cholera bacillus
Salmonella typhosa	*Bacillus typhosus* *Bacterium typhosum* *Salmonella typhi* *Eberthella typhosa* Typhoid bacillus
Salmonella enteritidis	*Bacillus enteritidis* *Bacterium enteritidis* *Salmonella enteritidis* "Gaertner" Gaertner bacillus
Salmonella gallinarum	*Shigella gallinarum* *Bacterium gallinarum* Fowl typhoid bacillus

Xylose was used for many years to differentiate *S. paratyphi* from other organisms of the genus. It has since been shown that while *S. paratyphi* never ferments this substance, many strains of *S. typhosa, S. typhimurium, S. anatis, S. newington,* and others decompose xylose.

The utilization of d-tartarate was believed to be a differential diagnostic means of distinguishing "human" from "animal" Salmonellas. Later examination demonstrated that many *S. schottmuelleri, S. typhimurium, S. pullorum, S. gallirarum,* and others do not follow this rule.

The only occasion where special biochemical reactions serve as a criterion for the identification of individual types occurs in the first members of the group "C" of Salmonellas and their monophasic varieties. In addition, biochemical reactions may help to distinguish *S. pullorum* from *S. gallinarum,* when the first does not produce an appreciable amount of gas or is slow in its production. Table 146 shows the biochemical reactions utilized in these cases.

ANTIGENIC STRUCTURE

The antigenic structure of the members of the genus *Salmonella* was presented in the White-Kauffmann scheme which had to be enlarged as newly discovered strains were described. New charts were published by Edwards and Bruner, Mickle, Bornstein, and others.

In order to elucidate the complex serologic structure of these bacteria, it must be taken into consideration that Salmonellas show different types of variation which affect their antigenic structure. The following is a brief review of these variations.

H—O Variation

The designations "H" and "O" originate from the observation of the spreading (H) and nonspreading (O) colonies of Protei. Subsequently these observations led to the study of the somatic and flagellar antigens of these and other organisms. It was established that "O" antigens are somatic antigens, resistant to heat and alcohol and giving a granular agglutination which develops best when the mixture of antiserum-antigen is incubated at higher, e.g., 50° C. to 56° C., temperature for several hours. The "H" antigens are flagellar antigens, thus present only in motile organisms; they are resistant to formol but not to heat or alcohol and give fluffy clumps in the agglutination test which takes place at lower temperatures, e.g., 37° C., and develops rapidly, usually within two hours.

The "O" antigens are designated by Roman numerals. The division of Salmonellas into groups "A," "B," "C," etc., is based upon these antigens. It

TABLE 146.—ANTIGENIC AND BIOCHEMICAL PROPERTIES OF SOME MEMBERS OF THE "C" AND "D" GROUP OF THE GENUS SALMONELLA

SALMONELLA	ANTIGENIC STRUCTURE			FERMENTATION OF		FORMATION OF H_2S
				Inositol	Trehalose	
S. hirschfeldi	VI, VII	c	1,5 . . .	0*	+*	+
S. hirschfeldi var. east africa	VI, VII, Vi	c	1,5 . . .	0	+	+
S. choleraesuis, biphasic, var. america	VI, VII	c	1,5 . . .	0	0	0
S. choleraesuis, monophasic, var. kunzendorf	VI, VII	-	1,5 . . .	0	0	+
S. typhisuis	VI, VII	c	1,5 . . .	0	+	0
S. typhisuis var. voldagsen	VI, VII	-	1,5 . . .	0	+	0
S. thompson	VI, VII	k	1,5 . . .	+	+	+
S. thompson var. berlin	VI, VII	-	1,5 . . .	+	+	+
				Dulcitol	Maltose	
S. pullorum	IX, XII	-	—	0	mostly +	+
S. gallinarum	IX, XII	-	—	+	+	+
S. gallinarum var. duisburg	IX, XII	-	—	+	+	0

*0 = fermentation absent; + = fermentation present.

was found to be necessary to divide some of these groups into subgroups. The group "F," also called "Further organisms," is steadily increasing with the discovery of new strains and with the acceptance of new "O" antigens as Salmonella antigens.

Some strains occasionally lack one of the "O" antigens which is usually present in their structure. While previous tabulations, mainly those of European workers, emphasize such deviations, recent authors regard them as mere variations. These antigens are enclosed in parentheses in American tabulations.

Form Variation

This term is used to designate the quantitative differences in a part of the somatic antigen. It usually involves antigens I, VI, and XII. Because of this variation, antigen XII has been alternately introduced and excluded from Salmonella tabulations.

V—W Variation

The antigen Vi (also called V) was originally discovered in inagglutinable cultures of *S. typhosa* and was thought to be connected with virulence. The Vi antigen was found to occur in *S. typhosa, Paracolobactrum ballerup,* the "East African" variety of *S. hirschfeldi,* and in some strains of *S. newport.* It is less resistant to heat than other somatic antigens. It may be preserved by dehydration at a temperature of 60° to 70° C. when its suspension in absolute alcohol is treated *in vacuo.* Colonies of organisms containing Vi antigen are slightly more opaque than the "W" colonies and have a ground glass appearance in reflected light. The loss of the Vi antigen is connected with the colony change from "V" to "W" form. While "V" colonies are susceptible to phage action, "W" colonies are not. The phage susceptibility of "V" forms is utilized in the typing of *S. typhosa* strains by the method of Craigie and Yen. While the susceptibility to phage action decreases during the V—W variation, the susceptibility of the strain to phagocytosis increases. An organism gradually losing its Vi antigen first shows a decrease in its ability to absorb Vi antibodies from anti-Vi sera, then a tendency toward reduction in agglutinability with Vi antiserum is demonstrable. Finally, the strain shows the typical "O" structure of the organism.

S—R Variation

When this phenomenon was first described, it applied to the colony form and to the stability of the growth in broth cultures. As the original smooth (S) growth on solid media becomes rough (R), the colonies appear to have a rough surface and irregular borders, and the broth cultures form a granular sediment. Slight grades of roughness may be discovered according to the method of Pampana by mixing a drop of a heavy suspension of the organism with a drop of 1:500 acriflavine. When the culture is rough, agglutination occurs.

The "R" form differs from the "S" not only in colony morphology but also in its loss of other characteristics, including specific "O" agglutinability, ability to absorb and to produce antibodies, toxicity for mice, biochemical activity, and other properties. In practical laboratory work, the greatest difficulties are encountered while "typing" strains which do not as yet reveal morphologic roughness but have become serologically rough. Such organisms are frequently encountered among colonies fished from strongly selective media, as the plate of Wilson and Blair, or among strains which have been maintained for a considerable period of time on carbohydrate media before antigenic analysis. A certain number of these strains can be serologically evaluated by using the technic of White which consists of suspending the growth from the surface of an agar slant in absolute alcohol, heating this mixture for one hour at 60° C., then draining and resuspending it in saline.

The "R" cultures contain a nonspecific somatic antigen, which seems to be common to groups or even to the entire genus Salmonella. A related genus-specific "R" antigen is present in rough Shigella strains. Thus, roughness of the culture renders it serologically nonspecific.

Rough cultures are often isolated from chronic carriers. Felsenfeld discovered two such cases which came under observation at a time when no more smooth forms but only rough colonies could be detected in the stools. Both carriers were found during surveys of food handlers. In the first case the organisms were isolated from the stools for a period of three weeks; in the second only one fecal specimen contained such bacteria. The rough colonies from both cases showed traces of the "O" antigen of group "B." All attempts to revert the cultures to the "S" form resulted in failure, so that it was impossible to establish the diagnosis of the types involved.

The variations described above concern solely the "O" antigenic structure and not the "H," with the exception of cases where the "R" variation has progressed so far that motility has been affected.

Phase Variation

The "H" antigens are flagellar antigens and therefore present only in motile cultures. When strains of motile Salmonellas are plated, two types of colonies are observed in most cases. One group of colonies possesses antigens which were originally considered specific for the type, while the other group of colonies shows antigens which are common to many Salmonellas and were, therefore, considered nonspecific. Thus the antigens characterizing the "phases" were called "specific" and "nonspecific," antigens of the first group being designated by small Latin letters and those of the second by Arabic numerals.

When the specific "H" antigens became so numerous that all the letters of the alphabet had been employed, later investigators found it necessary to designate newly discovered antigens by using index numbers attached to the letter "z," "z_1," "z_2," etc.

It has become an established custom to call the "specific" phase the "first" phase, the "nonspecific" the "second" phase. Kauffmann and Mitsui proved that certain antigens present in the first phase may also occur in the second phase. They introduced the terminology "alpha" and "beta" phases, which was subsequently eliminated. Edwards and Bruner succeeded in inducing artificial phases and accomplished much toward the reduction of the confusion which clouded the concept of phase variation. Seligmann and associates described a new phase, which they called "X." The importance of this phase has not as yet been clarified.

Salmonellas which occur only in one phase are known as "monophasic." Organisms found only in the first phase are called "monophasic, specific," while those present only in the second phase are termed "monophasic, nonspecific." Occasionally one of the phases may be weak and it is necessary to enhance it by using cultural and serologic aids. Edwards and Bruner recommended the addition of agglutinating serum to semisolid agar, thereby suppressing one phase and permitting the spread of organisms containing the other phase. Hajna described a simple modification of this technic. He uses U-shaped culture tubes which are filled with a mixture of Edwards and Bruner semisolid agar (M-84) with the addition of a small amount of agglutinating serum of the phase to be suppressed. The medium is inoculated with the culture by barely penetrating the surface of the agar in one branch of the tube. The phase which was not suppressed by the serum may be isolated from the growth in the uninoculated branch of the U-tube, after incubation.

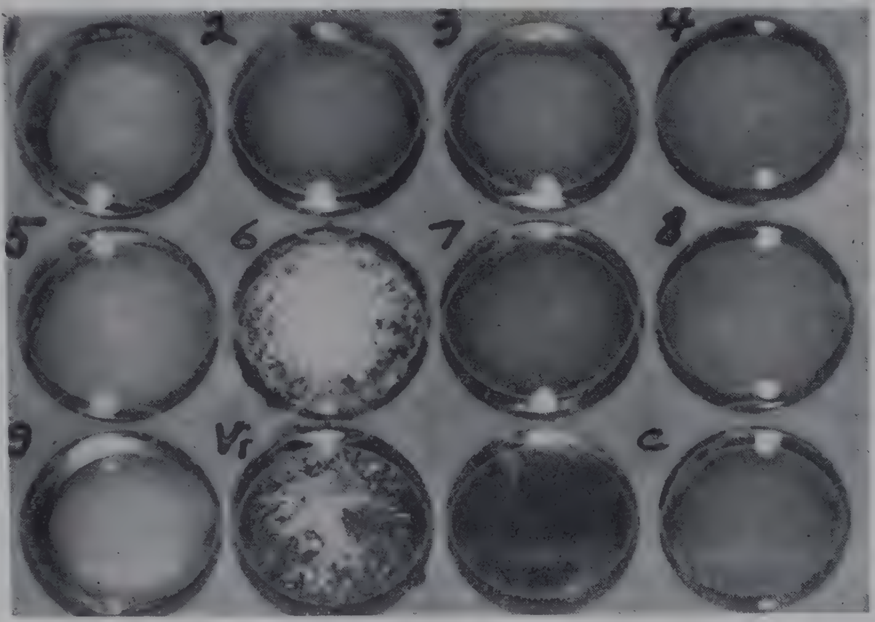

Fig. 362.—Slide agglutination tests involving *S. typhosa.* The numbers are the numbers of the sera ("O" sera) and the last is "Vi." "C" is the control. (Courtesy Dr. Oscar Felsenfeld.)

SALMONELLA TYPING

The antigenic properties and their variations are utilized practically in the serodiagnosis of Salmonellas, which is called "typing." The ideal method would be to have on hand single factor sera prepared against each antigenic component, and to test every strain with all of them. For practical purposes, however, it is not necessary to use such an elaborate system. For the typing of all known Salmonellas 31 "O" sera, one "Vi" serum and 39 "H" sera are utilized, from which single factor sera are prepared by absorption when necessary. Edwards and Bruner and Mickle have enumerated these sera and presented methods of preparation. For smaller laboratories the use of shorter series of sera was found satisfactory, giving special consideration to the frequency of the occurrence of Salmonella strains in different localities. Thus, *S. paratyphi* is rare in the United States; *S. enteritidis* is more frequent in Europe; *S. dublin* in East Africa and South America; *S. hirschfeldi* in the tropics.

It must be realized that many types are not brought to the attention of those bacteriologists who publish statistics. Many laboratories diagnose *S. typhosa, S. pullorum,* and *S. gallinarum* by simple means. Considerable scientific material is also being lost in laboratories which perform agglutinations only with sera against *S. typhosa, S. paratyphi,* and *S. schottmuelleri,* and report the results according to the highest agglutination titer, or discard the culture as ''negative'' if no agglutination occurs. With the advent of centers which are prepared to test any suspicious organism, the correct diagnosis can be easily ascertained. The most active Salmonella centers in America are: Communicable Disease Center, Chamblee, Georgia, the National Salmonella Center at Beth Israel Hospital in New York, N. Y.; the Institute of Hygiene, Montevideo, Uruguay, and others.

Even smaller laboratories may diagnose a large number of Salmonellas, using selected sera and keeping in mind that the types accounting for about 95 per cent of all isolated strains in the United States are those listed in Table 147.

TABLE 147.—FREQUENCY OF SALMONELLAE IN THE UNITED STATES, IN MAN

PER CENT OCCURRENCE	1939 TO 1945	1946 TO 1952
25 to 35	*S. typhimurium*	*S. typhimurium*
15 to 25	*S. schottmuelleri*	*S. enteritidis*
5 to 10	*S. typhosa* *S. oranienburg* *S. newport* *S. choleraesuis* *S. montevideo*	*S. typhosa* *S. oranienburg* *S. newport* *S. schottmuelleri* *S. montevideo*
1 to 5	*S. panama* *S. anatis* *S. enteritidis* *S. derby* *S. bareilly* *S. senftenberg* *S. give*	*S. panama* *S. anatis* *S. thompson* *S. derby* *S. bareilly* *S. choleraesuis* *S. tennessee*

The small laboratory should have two means for the serologic identification of Salmonellas: a polyvalent serum for the diagnosis of the genus by a single agglutination test and a few ''O'' and ''H'' sera which enable the bacteriologist to differentiate at least the most frequent strains.

Polyvalent sera have been described. Table 148 shows the preparation of two such sera. They are best used in test tube agglutination tests, incubating for two hours at 56° C. Seligmann (1947) described an excellent pooled polyvalent serum, which is preferred by many workers.

It is recommended that specific agglutinating sera be used for the ''O'' agglutination covering the members of the groups ''A'' to ''E.'' The preparation of agglutinating sera is best accomplished by adhering to the well-established method of Edwards and Bruner. This procedure is also followed here, incorporating slight modifications.

Table 149 shows the antigenic structure of the organisms used for the preparation of anti-''O'' sera in this abbreviated series. One rabbit is utilized for the production of each serum. The organisms are cultivated on the surface of nutrient agar slants for 24 hours; then the growth is washed off with saline and kept in the boiling water bath for two hours, to destroy "H" antigens. The animals are injected using 0.25, 0.5, 1.0, and 2.0 c.c. of the bacterial suspensions, at four-day intervals. Six days later the rabbits are bled.

The absorbing organisms are grown on nutrient agar plates. After 24 hours' incubation, the growth is washed off with saline, centrifuged, the supernatant liquid discarded and replaced with serum diluted 1:9 with saline. After 2 hours' incubation at 50° to 52° C., the suspensions are centrifuged and the supernatant fluid saved after adding 1:10,000 Merthiolate.

TABLE 148.—THE PREPARATION OF POLYVALENT SALMONELLA SERA

PREPARATION	TECHNIC ACCORDING TO	
	GALTON AND QUAN	FELSENFELD AND YOUNG
Organisms used as antigens	*S. paratyphi* *S. schottmuelleri* var. java *S. typhimurium* *S. thompson* var. berlin *S. oranienburg* *S. newport* *S. enteritidis* *S. anatis* *S. senftenberg* *S. worthington*	*S. paratyphi* *S. schottmuelleri* var. java *S. typhimurium* *S. hartford* *S. muenchen* *S. enteritidis* *S. london* *S. selandia* *S. senftenberg*
Formolized antigens injected	0.5, 0.5, 1 c.c.	0.5, 1.0, 2.0, and 2.0 c.c.
Alcoholized antigens injected	none	0.5, 1.0, and 2.0 c.c.
Live cultures injected	0.5 and 0.5 c.c.	0.5, 1.0, and 1.0 c.c.
Interval of injections	4 days	4 days

TABLE 149.—PREPARATION OF "O" AGGLUTINATING SERA

GROUP	ANTIGENIC STRUCTURE	ORGANISM USED FOR PREPARATION	NUMBER OF PLATES PER C.C. SERUM USED FOR ABSORPTION OF RAW SERUM	
			FIRST ABSORPTION	SECOND ABSORPTION
A	I, II, XII	*S. paratyphi*	*S. typhimurium* 5	*S. pullorum* 5
B_1	(I), IV, V, XII	*S. typhimurium*	*S. paratyphi* 5	*S. pullorum* 5
B_2	(I), IV, XXVII, XII	*S. bredeney*	*S. typhimurium* 5	*S. typhimurium* 5
C_1	VI, VII	*S. oranienburg*	*S. newport* 5	*S. newport* 5
C_2	(VI), VIII	*S. virginia*		
D	(I), IX, XII	*S. pullorum*	*S. paratyphi* 5	
E_1	III, XII, XXVI	*S. anatis*	*S. newington* 5	*S. newington* 5
E_2	III, XV	*S. newington*	*S. anatis* 5	*S. anatis* 5
E_3	(I), III, XIX	*S. senftenberg*	*S. newington* 10	*S. paratyphi* 5
	Vi	*S. ballerup* V	*S. ballerup* W 5	

For the performance of the agglutination tests, unabsorbed sera are diluted 1:5 and 1:10. Absorbed sera are used undiluted. Saline suspensions of the unknown cultures grown on agar serve as antigens. The cultures may be heated in the boiling water bath for one hour before testing. Slightly rough cultures must be treated with alcohol, according to the method of White described above in the section dealing with S—R variation.

The test is performed on a slide: a drop of the serum is mixed with a drop of the antigen, the slide tilted for about one minute and the results read. Table 149 shows the sera recommended for "O" agglutination tests.

More than 90 per cent of all Salmonellas will react with these "O" sera. If no agglutination occurs and the organism shows biochemical reactions typical for Salmonellas, the bacteria are tested with sera prepared against the group ''F,'' or, if such sera are not at hand, the strain is mailed to a Salmonella center for identification.

Both multiple and single factor sera are used for ''H'' agglutination tests. In order to prepare these sera, the organisms are cultivated in motility medium M-83 which assures the best production of the flagellar antigen. When necessary, the technic of Edwards and Bruner or of Hajna is used to elicit the suppressed phase, as described under ''Phase variation.'' Growth obtained from the bottom of a tube of Edwards and Bruner's medium, or from the uninoculated branch of the Hajna U-tube, is transferred into broth. After 18 to 24 hours of incubation the culture is diluted with an equal amount of 0.6 per cent formol containing saline: Rabbits receive 0.5, 1.0, 2.0, and 3.0 c.c. injections of these suspensions, utilizing the same technic as described for the preparation of ''O'' sera.

In order to produce single factor sera, the technic of Edwards and Bruner is used to absorb the multiple factor sera. The organisms utilized for the absorption are grown on nutrient agar plates, 10 cm. in diameter. After 24-hour incubation, the growth is washed off with sufficient saline to give 0.5 c.c. of a bacillary suspension from each plate. The growth from several (usually 5 to 10) plates is added to each c.c. of serum which is diluted 1:9 with saline, and the mixture incubated for two hours at 50° C. The organisms are then removed by centrifugation. In the ''double absorption technic'' two consecutive absorptions are carried out. Table 150 presents the organisms used for the preparation of such single factor sera, giving the number of plates used for the absorption as well as those sera which are used to suppress phases of Salmonellas. Only those sera are included in the tabulation which are considered essential for a small laboratory.

TABLE 150.—PREPARATION OF ''H'' AGGLUTINATING SERA

A. Basic Sera

ANTIGEN	ORGANISMS USED TO INJECT RABBITS FOR THE PRODUCTION OF SERUM	ORGANISM USED MUST BE CULTIVATED IN THE PRESENCE OF ''H'' SERUM (TO SUPPRESS UNDESIRED PHASE) AGAINST
a	*S. paratyphi*	
b	*S. minnesota,* monophasic	
c	*S. choleraesuis,* 1 phase	*S. reading*
d	*S. typhosa*	
i	*S. bonariensis,* 1 phase	*S. abortus equi*
eh	IV.V:eh*	
cnx	*S. abortus equi*	
fg	*S. adelaide*	
gms	*S. montevideo*	
k	*S. thompson*	*S. choleraesuis* var. *kunzendorf*
lv	*S. bredeney,* 1 phase	
mt	*S. oranienburg*	
y	XXVIII:y*	
1,2	*S. newport* var. *puerto rico*	
1,5	*S. choleraesuis* var. *kunzendorf*	
1,6	*S. anatis,* 2 phase	*S. reading*
1,7	*S. give,* 2 phase	*S. panama*
z_{29}	*S. tennessee*	

B. Single Factor Sera

FACTOR DESIRED	SERUM TO BE ABSORBED	NUMBER OF PLATES PER C.C. OF SERUM USED TO ABSORB			
		FIRST ABSORPTION		SECOND ABSORPTION	
f	*S. adelaide*	*S. montevideo*	5	*S. enteritidis*	5
m	*S. oranienburg*	*S. senftenberg*	3	*S. berta*	7
s	*S. montevideo*	*S. enteritidis*	5	*S. enteritidis*	5
t	*S. oranienburg*	*S. montevideo*	5	*S. montevideo*	5
v	*S. bredeney,* 1 phase	*S. worthington*	5	*S. worthington*	5
2	*S. newport* var. *puerto rico*	*S. bredeney*	5	*S. thompson*	10
5	*S. choleraesuis* var. *kunzendorf*	*S. anatis*	5	*S. newport*	5
6	*S. anatis,* 2 phase	*S. muenster*	5	*S. thompson*	10
7	*S. give,* 2 phase	*S. newport*	5	*S. panama*	5

*Monophasic unnamed organisms.

Tube agglutination tests are used to determine the ''H'' antigens. Kauffmann originally recommended the performance of slide agglutination tests for the determination of both ''O'' and ''H'' antigens. Experience has demonstrated that test tube agglutinations are more reliable because cross reactions which frequently occur among ''H'' factors are more easily eliminated. The titer of the sera for typing of ''H'' antigens must be higher than 1:1,000, so that ''O'' antigens do not interfere with the reaction. For this reason the sera are used in dilutions near their final titer. Equal amount of diluted serum (usually 1:1,000) and formolized antigens are used in single tube tests.

The unknown organism is grown in nutrient broth and, after 24 hours, 1 or 2 drops of concentrated Formalin are added. The tests can be carried out after 3 hours or more. The reactions are read after a few minutes and again after one hour's incubation at 50° to 52° C. in a water bath.

When the unknown Salmonella shows the reactions of only one phase, attempts should be made to enhance the other phase, except in cases where a group of ''H'' antigens was found which occurs exclusively in monophasic types.

Antigens f, g, m, s, and t are present mostly in the first phase only.

Single-factor 2, 5, 6, and 7 sera are preferred to unabsorbed 1,2; 1,5; 1,6; and 1,7 sera.

Recent simplifications have discarded antigens 3, 4, 10, 11, o, z_1, z_2, z_8, z_9, z_{17} as diagnostic factors. Furthermore, *Salmonella ballerup* (and the related *S. hormaeche* and *S. exeter* types), as well as *S. arizona*, have been taken off the list of Salmonellae. They are now members of the Ballerup and Arizona groups, respectively, of paracolon organisms.

Lists of Salmonellae are never up to date. It is felt, therefore, that the names of the 286 Salmonellae known at this writing should not be included in this textbook, since extensions of the list would be necessary every month or so.

Only a simplified schedule of typing is given here. This procedure must be modified, especially outside the United States, to suit local conditions. The addition of p, q, and u factors is recommended in areas where *S. dublin* (IX, XII: g, p), *S. rostock* (IX, XII: g, p, u), and *S. blegdam* (IX, XII: g, m, q) are present.

If the tested organism reacts with only one serum recommended for use in the particular group, the strain must be cultured in the presence of that serum (using the Edwards and Bruner or Hajna technic, see pages 1522 ff.) and then re-tested with the other sera.

If a complete diagnosis cannot be made by using this simplified schedule, the strain should be mailed, without delay, to a Salmonella typing center. There is no use in wasting time and sera with futile experiments. Delay in submitting cultures to a better equipped laboratory results in the cultures' turning rough, which in turn makes typing difficult or even impossible.

Simplified Schedule of Salmonella Typing

It must be emphasized that all organisms which cannot be easily classified in the laboratory must be forwarded to a typing center.

This simplified schedule does not contain Salmonella groups beyond E_3, since at least 95% of the Salmonellae frequently encountered in the United States belong to groups B, C, D, and E. The polyvalent sera do not contain ''O'' factors for further group testing, and thus the agglutination tests with this serum will be negative.

I. If the organism shows the biochemical properties of *S. typhosa*—is motile; forms only acid but not gas from dextrose and mannitol; forms some hydrogen sulphide; does not produce indol, urease, or methyl ethyl carbinol; does not ferment lactose or sucrose—test first with ''D,'' ''Vi,'' and ''d'' sera.

If there is no agglutination, test with Salmonella polyvalent serum, since it may be an anaerogenic Salmonella.

II. If the organism shows the biochemical properties of a Salmonella without producing hydrogen sulphide, test with polyvalent Salmonella serum first. Hydrogen sulphide-negative Salmonellae are rare in the United States. They are frequent in the Old World (*S. paratyphi*, *S. choleraesuis* biphasic variety, *S. sendai*, etc.). In animals, *S. abortus equi* is often present.

III. Organisms giving Salmonella biochemical reactions and which are nonmotile must be suspected of being *S. pullorum.* This will react with ‘‘D’’ serum. *S. pullorum* is rare in man.

IV. In all other cases—motile organism which forms hydrogen sulphide, acid and gas from dextrose and mannitol; no indol, urease, or methyl ethyl carbinol; does not ferment sucrose or lactose—test with polyvalent ‘‘O,’’ then ‘‘B_1,’’ ‘‘C_1,’’ ‘‘C_2,’’ ‘‘D,’’ and raw ‘‘E’’ sera.

If there is no reaction, repeat with alcoholized antigen.

If there is still no reaction, put organism into stock agar (M-6 or M-15) for eventual mailing; streak a purity plate; pick again and inoculate into: lactose, sucrose, salicin, dulcitol, and adonitol.

If lactose, sucrose, or salicin are not fermented, submit strain to a Salmonella typing center.

Arizona group paracolons usually do not ferment any of these five substances in one week; however, they usually break gelatin.

Ballerup group paracolons only exceptionally ferment dulcitol but will break (with one or two exceptions) one or more of the lactose-sucrose-salicin series, but not adonitol.

Bethesda group paracolons usually ferment dulcitol rapidly and will decompose one or more of the other substances except adonitol which is, however, usually attached by *Providence* paracolons. This latter group is usually anaerobic and mannitol negative. *Providence* organisms differ from Protei by their lack of urease.

V. If a strain is agglutinated with polyvalent ‘‘O’’ serum and produces hydrogen sulphide, the following procedure is recommended:

(a) Test with ‘‘B_1’’ serum. If agglutinated, test with ‘‘B_2’’ serum:
(1) If ‘‘B_2’’ negative, test with ‘‘b,’’ ‘‘i,’’ and ‘‘2’’ sera.
b+ 2+ : *S. schottmuelleri*
i+ 2+ : *S. typhimurium*
If b– i– : test with ‘‘fg,’’ ‘‘eh,’’ ‘‘mt,’’ ‘‘5’’ sera.
fg+ mt– : *S. derby*
fg+ mt+ : send to typing center (may be *S. california, essen,* etc.)
eh+ 2+ : *S. saint paul*
eh+ 5+ : *S. reading*
(2) ‘‘B_2’’ positive: test with ‘‘b,’’ ‘‘lv,’’ ‘‘7,’’ ‘‘enz_{15}’’ sera.
b+ enz_{15}+ : *S. abortus bovis*
kv+ 7+ : *S. bredeney*

(b) Agglutinated with ‘‘C_1’’ serum: test with ‘‘c,’’ ‘‘fg,’’ ‘‘mt,’’ ‘‘5’’ sera.
c+ 5+ : proceed according to Table 147
fg+ mt+ : *S. montevideo* (g, m, s)
fg– mt+ : *S. oranienburg* (m, t)
Confirm with absorbed sera.
If c– fg– mt– : test with ‘‘k,’’ ‘‘y,’’ ‘‘g,’’ and ‘‘z_{29}’’ sera.
k+ 5+ : proceed according to Table 147
y+ 5+ : *S. bareilly*
z_{29} : *S. tennessee*

(c) Agglutinated with ‘‘C_2’’ serum: test with ‘‘eh,’’ ‘‘d,’’ and ‘‘2’’ sera.
eh+ 2+ : *S. newport*
d+ 2+ : *S. muenchen*

(d) Agglutinated with ‘‘D’’ serum but not *S. typhosa* or *S. pullorum*: test with ‘‘fg,’’ ‘‘gms’’ sera. If positive either in one or in both, test single factors f, m, s, t:
fg+ gms+ f– m+ s– t– : *S. enteritidis* (gm) or *S. blegdam* (gmq) (in the Far East)
f– m– s– t– : send to Salmonella center (may be *S. dublin, S. rostock, S. moscow*)
t+ : send to Salmonella center (may be *S. pensacola* or *S. berta*)
If fg– gms– : test with ‘‘a,’’ ‘‘lv,’’ and ‘‘5.’’
a+ 5+ : *S. sendai* (Far East), *S. miami*
lv+ 5+ : *S. panama* (confirm with single factor ‘‘l’’ serum)

(e) Agglutinated with raw ''E'' serum. Use absorbed ''E_1,'' ''E_2,'' and ''E_3'' sera.
(1) E_1 positive: test with ''eh,'' ''lv,'' ''5,'' ''6,'' and ''7'' sera:
eh+ 5+ : *S. muenster*
eh+ 6+ : *S. anatis* (frequent)
eh+ 7+ : *S. nyborg* (rare)
lv + 5+ : retest with ''v.'' If negative, *S. uganda* (rare, lz_{13}–1,5)
lv + 6+ : *S. london*
lv + 7+ : *S. give*
eh+ lv+ : test with ''v'' and ''w.'' If v– w+ : *S. meleagridis* (e, h, – l, w).
(2) E_2 positive (rare): test with ''eh,'' ''6,'' and ''7'' sera.
eh+ 6+ : *S. newington*
eh+ 7+ : *S. selandia*
lv + 7+ : *S. new brunswick*
eh+ lv+ : test with ''v.'' If negative: *S. cambridge.*
(3) E_3 positive: test with ''s, t'' serum.
s, t+ confirm with ''g''; if +: *S. senftenberg.*

The use of this short method will establish the diagnosis of about 95 per cent of the strains found in man in the United States. Infrequently encountered organisms or those which do not fit into the established scheme must be mailed to a more completely equipped center.

Care should be exerted in the interpretation of the findings, because of the many cross reactions and ''minor antigens.'' As a rule, a higher dilution of the ''H'' antisera excludes ''minor antigens'' or those antigens which were not completely eliminated by absorption. The difficulties experienced with antigens I, VI, and XII have already been mentioned. Antigens VIII and XI also display a trend toward partial cross reactions. The problem is further complicated by the occurrence of Salmonella antigens in other organisms, mainly in closely related coliform and paracolon bacilli. Such organisms may show parts of Salmonella ''O'' or even ''H'' antigens, especially I, II, V to VII, XII to XIV, XVI, XIX, XXIII to XXX; VI; z_4, z_{23}, z_{24}, 1,5 . . .etc. No explanation has been found as yet for the occurrence of such serologic reactions. It is believed

TABLE 151.—VARIETIES NOT INCLUDED IN RECENT TABLES UNDER OWN NAME

STRAIN	VARIETY
S. paratyphi	durazzo
S. schottmuelleri	odense java ''animal strain''
S. typhimurium	copenhagen binns
S. hirschfeldi	east africa
S. choleraesuis	kunzendorf
S. typhisuis	voldagsen
S. thompson	berlin
S. enteritidis	danysz chaco essen
S. gallinarum	duisburg
S. senftenberg	newcastle
S. newport	puerto rico pueris
S. sendai	miami
S. muenchen	oregon mexicana
S. saint paul	zagreb

TABLE 152.—THE REACTIONS OF "ENTERIC" MICROORGANISMS IN DIFFERENTIATING MEDIA

GROWTH IN						REACTION		FERMENTATION OF		
Triple sugar iron medium (M-80)			Semisolid mannitol (M-88)							
Slant	Butt	H_2S formed	Fermentation	Motility	Urease (M-90)	Indol (R-12)	Voges Proskauer (R-14)	Rhamnose (M-5)	Dulcitol (M-5)	ORGANISM
k*	AG*	V*	AG	+*	0*	0	0	x*	x	Salmonella
k	AG	+	AG	0	0	0	0	x	0	*S. pullorum*
k	AG	V	AG	V	V*	V	V	x	x	Paracolon
k	AG	+	0	+	+	+	0	x	x	*Pr. vulgaris*
k	AG	+	0	+	+	0	0	x	x	*Pr. mirabilis*
k	AG	0	0	+	+	+	0	x	x	*Pr. morganii*
k	A*	0	A	+	+	+	V	x	x	*Pr. rettgeri*
k	A	sl*	A	+	0	0	0	x	x	*S. typhosa*
k	A	0	0	0	0	0	0	0	0	*Sh. dysenteriae*
k	A	0	A	A-E	0	V	0	0	0	*Sh. paradysenteriae* group
k	A	0	A	0	0	+	0	+	V	*Sh. alkalescens dispar* group
k	A	V	A	0	0	0	0	0	+	*S. gallinarum*
k	A	0	A	0	0	0	0	+	0	*Sh. sonnei*
k	A	V	A	V	V	V	V	x	x	*Paracolon anaerogenes*
A	A	0	x	x	0	x	x	x	x	Gram +
A	AG	V	x	x	0	x	x	x	x	Coliform
k	k	0	x	x	V	x	x	x	x	Alcaligenes
k	k	+	x	x	V	x	x	x	x	Pseudomonas

*K = alkaline; A = acid produced; AG = acid and gas produced; sl = slight reaction; lw = slow reaction; V = variable reaction; x = not necessary for the determination of this organism; + = positive reaction; 0 = no reaction. E = *Sh. paradysenteriae* 4 c (rabaulensis and rio) does not ferment mannitol. N = negative, except *Sh. paradysenteriae* Flexner 6.

that these organisms could originate from Salmonellas possessing more complex antigens by loss of certain serologic factors. Salmonella antigens, however, were found not only in closely related Enterobacteriaceae. It was discovered that *Pasteurella pseudotuberculosis* contains part of IV, V, XII, *Pasteurella avicida,* the Vi, and *Pneumococcus* type 35 antigen XVII of the Salmonellas. There is, perhaps, a chemical relationship of haptens, similar to that existing between the antigens of OX protei and Rickettsiae.

Edwards described monophasic strains which greatly facilitate the production of single factor Salmonella sera.

Lederle, Inc., began the production of Salmonella typing sera on a commercial basis. These sera (polyvalent; group sera for groups A, B, C_1, C_2, D, and E; Vi and d sera) facilitate the typing procedure.

The phage typing of Salmonellae was attempted but is not being carried out as a routine diagnostic means.

AGGLUTINATION TESTS FOR TYPHOID AND SALMONELLA INFECTIONS

It is unfortunate that many physicians ask for an agglutination test for paratyphoid A in the Northern part of the United States, where this infection is practically nonexistent. Little attention is paid, as a rule, to agglutination tests for the most frequently found Salmonellae, as *S. typhimurium, S. montevideo,* or *S. oranienburg.* Most laboratories use typhoid, paratyphoid A and B antigens for the diagnosis of "enteric" infections. The preparation and use of such antigens is, therefore, given first.

The Macroscopic Widal Test

The example given below is for the diagnosis of typhoid fever. If any other disease is under consideration, the technic is the same, but a suspension of the bacteria causing the disease in question is used instead of a killed culture of typhoid bacilli.

Suspension of Killed Typhoid Bacilli

Use a culture of *Salmonella typhosa* that has been attenuated by at least several weeks' frequent transplantation on artificial culture media.

Inoculate bottles of nutrient broth with this culture and incubate at 37° C. for twenty-four hours (M-2).

To every 10 c.c. culture, add 0.1 c.c. of 10% solution of 40% formaldehyde. (Ten parts of 40% formaldehyde [Formalin] made up to 100 c.c. with distilled water.)

Place in the refrigerator and shake vigorously several times each day. Allow to remain in the refrigerator for at least four days. At the end of this time, the suspension is sterile. Remove any clumps by filtration through a layer of sterile absorbent cotton in a sterile funnel, catching the filtrate in a sterile bottle. This suspension will be useful as long as it shows no evidence of spontaneous agglutination.

Technic.—

Obtain clotted blood. Loosen the clot and centrifuge for 15 minutes at high speed. Decant the supernatant serum, placing it in a sterile corked test tube. Add 1 c.c. of this serum to 9 c.c. sterile physiologic saline. This is a 1:10 dilution of patient's serum.

Place Wassermann test tubes in a rack. Label them 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and control.

Add to tubes 2 to 7, inclusive, 0.5 c.c. saline solution. No saline is placed in tube 1.

Place in tubes 1 and 2, 0.5 c.c. of the 1:10 dilution of patient's serum. Mix the contents of tube 2 by drawing in and out of a pipette, withdraw 0.5 c.c., and place in tube 3. Mix the contents of tube 3, and remove 0.5 c.c. from this tube, placing it in tube 4. Continue through tube 6. When the contents of tube 6 have been thoroughly mixed, withdraw 0.5 c.c. and discard. Tube 7 is the saline control, and receives no serum. Add to each tube 0.5 c.c. of the suspension of killed typhoid bacilli (above). This makes the dilution of patient's serum in each tube as follows: 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640.

Mix the contents of each tube by shaking thoroughly.

Place the rack in the incubator for eight to twelve hours.

A positive reaction is indicated by the formation of a sediment consisting of agglutinated bacteria. The supernatant liquid is clear. The control tube should remain cloudy without a sediment. All negative tubes will be cloudy without a sediment. Report the dilutions at which positive reactions are obtained. Killed suspensions of many bacteria may be purchased from most biological houses.

Rapid Agglutination Test in Typhoid and Salmonella Fever, Typhus, and Brucellosis

Comparative results of the rapid slide test technic with a standard test tube method have been reported by Welch and Stuart[1] and Welch and Mickle.[2] In general, it can be stated that the rapid slide method possesses advantages over the test tube method. Tests can be made and reported on the same day the specimen is received. This is very important where a rapid method is essential regarding disposition of cases in hospitals.

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[1] J. Lab. & Cin. Med. **21**: 411, 1936.

[2] Am. J. Pub. Health **26**: 248, 1936.

Materials.—

Febrile Antigen Kit.*—The Lederle antigens contain droppers which deliver approximately 0.03 c.c. per drop.

Standard serologic pipettes. 0.2 c.c. capacity graduated in 0.001 c.c. These are the pipettes used in the Kahn test for syphilis.

Glass slides containing six rows of five each **wax rings** (see page 1007).

Technic.—

Use the first two rows of rings for ''O'' and ''H'' typhoid antigens, the third row for paratyphoid ''A,'' the fourth row for paratyphoid ''B,'' the fifth row for Proteus OX19, and the sixth for *Brucella abortus* antigen.

It is well to make both positive and negative controls on the antigens.

The same technic is used for each antigen.

Pipette serum into the various rings in the following quantities, beginning at the left and proceeding toward the right of the slide:

0.08 c.c., 0.04 c.c., 0.02 c.c., 0.01 c.c., and 0.005 c.c., using a Kahn pipette, 0.2 c.c. capacity, graduated in 0.001 c.c.

This gives serum concentrations, when mixed with the 0.03 c.c. of antigen of the following dilutions: 1:20, 1:40, 1:80, 1:160, and 1:320. If necessary, further dilutions may be made.

Add a drop of typhoid ''O'' antigen to each drop of serum in the first row, typhoid ''H'' antigen to the sera in the second row, paratyphoid ''A'' antigen to the third row, paratyphoid ''B'' to the fourth row, Proteus OX19 antigen to the fifth row, and Brucella antigen to the sixth row. Shake the antigens well but gently before using.

Using a separate toothpick, mix thoroughly each row of serum-antigen mixture, beginning with the smallest amount of serum and working from right to left.

Gently rock the glass slide back and forth 20 to 30 times and check the degree of clumping.

Record as ++++, complete clumping with no free antigen; +++, 75% clumping with 25% free antigen; ++, 50% clumping and 50% antigen; +, 25% clumping and 75% antigen; and ±, faint clumping. Absence of clumping is a negative reaction.

Clumping with ''O'' antigen differs from the typical small-flaking or granular agglutination obtained in the test tube method. ''O'' and ''H'' agglutination cannot be differentiated by appearance.

Interpretation of Results.—

A test may be positive for typhoid fever if it shows agglutination with either one or both of the typhoid antigens.

A positive agglutination with typhoid, paratyphoid ''A,'' or paratyphoid ''B'' antigens in a dilution of 1:80, if the patient has not had vaccine, is significant.

Cases of typhoid fever may show cross-agglutination with paratyphoid ''B'' antigen, but rarely with paratyphoid ''A,'' provided the patient has not had vaccine or previous infection with the Salmonella group of bacteria. If the patient has been vaccinated, or has history of previous infection, the results must be interpreted accordingly. In most vaccinated individuals, the results cannot be explained at all.

If the patient has been immunized with triple vaccine, agglutination with typhoid ''H'' and both paratyphoid ''A'' and ''B'' antigens will be expected for at least one year. ''O'' antigen may also show agglutination. ''O'' agglutinins usually disappear first; the ''H,'' ''A,'' and ''B'' agglutinins may persist even after three years.

Single specimens on an individual may be misinterpreted since cross reactions do occur with some sera.

Reporting the Results.—

Report to the physician the weakest concentration of the serum which causes agglutination of the antigen, and the antigen or antigens for which the reaction is specific.

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*Febrile antigens for this test may be secured from Lederle Laboratories, New York.

The test may show agglutination with both "O" and "H" antigens, depending on the time the blood serum is obtained. "O" agglutinins often appear in the patient's serum before "H" agglutinins, and "H" agglutinins often remain longer than "O" agglutinins. After typhoid immunization, the "H" agglutinin may be present for three years, and during the first year, some "O" agglutinins will appear. "O" agglutinins are more likely to be present if the individual has received more than one series of inoculations. For the most part, all agglutinins disappear from the blood stream within 5 to 10 years after immunization unless the individual has more than one series of inoculation.

Other Technics

(a) Brumpt.[1]—

Brumpt used whole blood which is permitted to dry on a slide.

The antigens are prepared by the addition of 10% sodium citrate, 0.2% formol, and 1 to 2 drops of a saturated watery solution of methylene blue *or* Sabrazes blue.

Salmonella, Proteus, Brucella, and *Pasteurella tularensis* antigens may be used in this way.

One drop of antigen is mixed with the drop of blood, and the reaction observed.

If liquid blood is available, serial dilutions, similar to those used by Welch and Stuart, are used. Different amounts of the antigen are put on a glass plate or cardboard and drops of blood added directly from the finger or ear or from the vein. When blood and antigen are mixed, the plate is rocked and the reactions read.

(b) Bagilova.*—

One-tenth c.c. amounts of suspensions containing 80 to 100 milliards of Protei, Salmonellae, etc., per c.c. are dried on pieces of cellophane paper. The papers are kept in a refrigerator and just before use are dissolved in 1 c.c. saline.

Such antigens keep for 3 months.

Vi Agglutination

The Vi agglutination has attracted much attention. It is considered an excellent aid for the detection of typhoid carriers. Unfortunately, no two authors have performed this test alike. Brower divided the methods for the detection of the Vi antibodies in the serum into the following groups:

1. **Absorption technic,** used by Felix.—The serum is first absorbed with a *S. typhosa* strain rich in "O" and "H" antigens, then the Vi titer is determined by agglutination with a Vi strain.
2. **Direct titration of antibodies** with a strain which contains only Vi antigens; for example, the Bhatnagar strain of *S. typhosa.*
3. **Direct titration** with a strain from which the "O" antigens have been eliminated by growing in broth containing "O" serum and the "H" antigens separated by repeated centrifugation and washing, according to the method of Detre.
4. **Precipitation tests,** using antigenic extracts from Vi strains.

Because of technical difficulties involved in the other methods, the third method was found to be the most satisfactory. It requires, however, considerable work. The cultures must be tested and transferred frequently and their V—W variation cannot be prevented easily.

The technic of the Vi routine agglutination test is that of tube agglutination tests. The dilutions of the sera, however, are lower: 1:5, 1:10, 1:20, and 1:40. A suspension of the Bhatnagar Vi strain in 0.85% sodium chloride is used. The antigen is adjusted to tube 3 of the McFarland nephelometric standard (page 1477). The tubes are incubated for 2 hours at 37° C., and then left in the refrigerator overnight. A hand lens is used to facilitate the reading.

Gunther[2] devised an excellent modification of Desranleau's[3] technic.

Grow Bhatnagar Vi strain on veal infusion agar M-15, pH 7.6, for 24 hours.

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*Zhurnal Mikrobiol. 1: 36, 1942.

[1] Bull. Soc. Path. Exot. 33: 390, 1940.

[2] Am. J. Clin. Path. 16: 293, 1946.

[3] Canad. Pub. Health J. 34: 502, 1943.

Wash off the growth with 10 c.c. of 75% alcohol per plate.

Keep in the refrigerator for 4 days, then centrifuge and wash with distilled water.

Suspend the sediment in 50% glycerol in 0.1% sodium chloride, buffered to pH 7.4 to 7.6.

Adjust the suspension to 10 times tube 1 of the nephelometer.

Dilute the serum 1:2 with saline.

Add to 0.05 c.c. of this dilution 0.05 c.c. of the antigen on a hollow slide.

Shake for 4 minutes on the Kahn shaker.

Read after 15 and 30 minutes using 150× magnification.

Most authors agree that the Vi agglutination test should be considered positive when agglutination occurs in tubes containing at least 1:20 dilution of serum. Positive tests were observed in a large proportion, about 80 per cent, of typhoid carriers, but agglutination is also found in about 5 to 10 per cent of the general population.

Shigella (Dysentery Bacilli)

Gram-negative, nonmotile, nonsporeforming rods, which do not give a Voges-Proskauer reaction; ferment dextrose with the formation of acid only; do not produce hydrogen sulphide. Do not utilize citrates as a sole source of carbon.

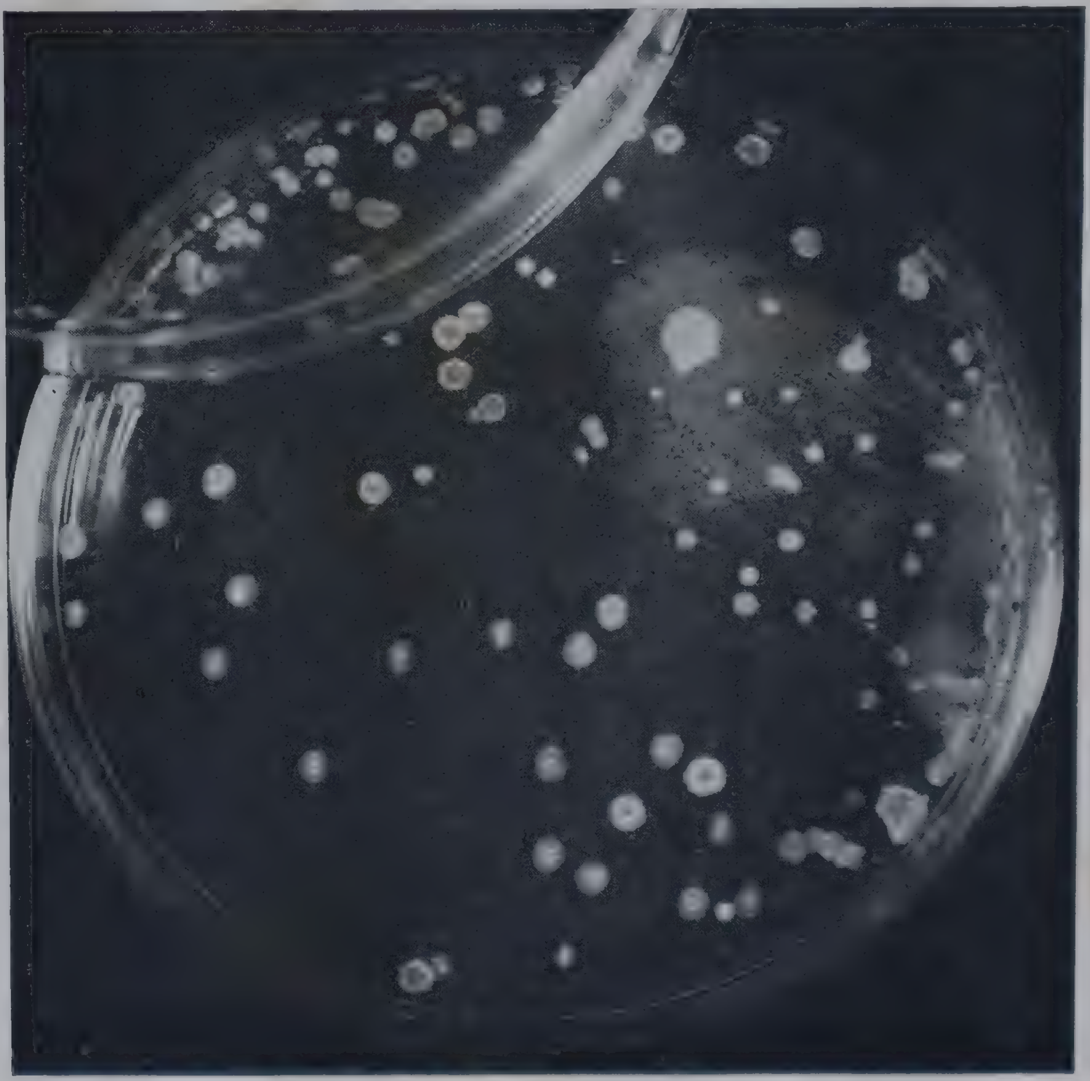

Fig. 363.—*Shigella sonnei* colonies isolated from stool specimen on SS agar. (Courtesy Dr. Oscar Felsenfeld.)

They grow well on the usual media, with the exception of a few strains which are fastidious (*Sh. dysenteriae*, rare members of the *Sh. paradysenteriae* group, very few *Sh. sonnei* strains).

The principal biochemical differences of strains important in human disease are given in Table 152.

Much information on the classification of Shigellae is contained in Neter's excellent review.[1] Other extensive articles were later published by Wheeler[2] and Weil.[3] The classification of the mannitol-negative strains was clarified by Wheeler and Stuart[4] who arrived at the following conclusions:

In the mannitol-negative, lactose-negative, and indol-negative group are *Sh. dysenteriae* Shiga-Kruse and Sachs' strains Q 454, Q 771 (identical with Gober's, ''8524'' and *Sh. arabinotarda* A, and Q 1167 (identical with *Sh. arabinotarda* B).

In the mannitol-negative, lactose-negative, and indol-positive group are the *Sh. ambigua* Schmitz, Sachs' strains Q 902 and Q 1030, and the new serotype 599-52.

The serologic classification of the strains is as follows:

Sh. dysenteriae, the true dysentery bacillus of Shiga-Kruse, is serologically homologous; one serum will agglutinate all strains. Serologically different strains (''Sachs'' strains), are, however, found. This is the only Shigella which forms a true exotoxin. It is rarely found in the United States of America.

Sh. ambigua, relatively rare in the United States, differs from *Sh. dysenteriae* in that it forms indol.

Sh. flexneri was formerly classified according to the schema of Andrewes and Inman as V, W, X, Y (= Hiss-Russell) and Z (= Strong), and combined forms, such as VZ, etc. Table 153 shows the more important forms according to the classification of Wheeler, Boyd, and Haden. *Sh. flexneri* is a frequent cause of dysentery.

Sh. alkalescens has at least 8 serologic types. It is one of the frequently found Shigellae.

Sh. sonnei is being reported in great numbers, principally in children and in inmates of asylums. It usually causes a short-term diarrhea, without frank blood in the stools. Two serologic types are recognized.

Sh. dispar has been differentiated into *Sh. ceylonensis* and *Sh. madampensis* on the basis of fermentation of dulcitol. Carpenter distinguished at least four serologic types. Recently it has been added to the alkalescens group under the name Alkalescens-Dispar.

SEROLOGY

It is not necessary to produce one's own sera since they are commercially available from Lederle Laboratories. These sera must, however, be used in strict adherence to the manufacturer's instructions. If sera are produced in the laboratory, smooth strains are selected and gown on extract agar slants M-13.

When injecting animals, give the first injection subcutaneously, the others intravenously. Inject the animals twice a week, for 5 weeks. Wash off the growth on the slants with sterile saline and kill by heating to 100° C. for 2 hours. Then add enough Formalin to make a 0.5% concentration. For the first injections, adjust to match tube 1 of the nephelometer; for later injections, adjust to tube 3.

Schedule of injections:

First injection: 0.1 c.c. of No. 1 density, subcutaneously.

Second to fifth injections: 0.1 c.c., 0.2 c.c., 0.5 c.c., 0.5 c.c. of No. 1 density intravenously.

Sixth to ninth injection: 0.5 c.c., 0.7 c.c., 1.0 c.c., and 1.0 c.c. of density No. 3 intravenously.

Bleed the animals after a 5 to 7 days' wait.*

The polyvalent serum for the A Group is prepared by pooling *Sh. dysenteriae* 1 to 7 type sera, according to Madsen,[5] and absorbing with Alkalescens-Dispar organisms.

The polyvalent serum for group B is prepared by pooling *Sh. flexneri* 1 to Y type sera and absorbing it with Alkalescens-Dispar 1, 3, and 4 organisms.

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*Some authors recommend shorter schedules to avoid panagglutinating seras, e.g., 0.5, 1.0, 2.0, 4.0, and 4.0 c.c. amounts in 4- to 5-day intervals.

[1] Bact. Rev. **6**: 1, 1942.

[2] J. Bact. **49**: 87, 1944.

[3] J. Immunol. **49**: 321, 1944.

[4] J. Bact. **51**: 317, 1946.

[5] Madsen: The Classification of the Shigella Types, Copenhagen, 1949, Ejnar Munksgaards Forlag.

The polyvalent serum for group C is prepared by pooling *Sh. boydii* 1 to 7 type sera and absorbing it with Alkalescens-Dispar 1, 3, 4 and *Sh. sonnei* II organisms.

The bivalent *Sh. sonnei* serum is a pool of sera against smooth and rough *Sh. sonnei* types.

The *Alkalescens-Dispar* group has at least 8 "O" groups and a polyvalent serum should represent the 5 most frequent types (*Sh. alkalescens,* I, II, III; *Sh. dispar* I, and Frantzen's strain 145 or 5513, the last being serologically identical with Kauffmann's *Escherichia coli* U 9/41).

Type-specific *Sh. flexneri* sera are not easy to prepare. In view of the diagnostic antigenic formulas of Madsen, the following absorptions are recommended:

Type 1 serum: absorb type 1a serum with type 2a, 5, and 6 organisms.
Type 2 serum: absorb type 2b serum with type 1a and X organisms.

TABLE 153.—CLASSIFICATION OF SHIGELLAE*

GROUP	OLD DESIGNATION	INTERNATIONAL NOMENCLATURE	LACTOSE	MANNITOL	INDOL	RHAMNOSE	DULCITOL
A	*Sh. dysenteriae*	*Sh. dysenteriae*					
	Shiga	type 1	0	0	0	0	0
	Sh. ambigua						
	Schmitz	type 2	0	0	+	+	0
	Sachs Q 771	type 3	0	0	0	0	0
	Sachs Q 1167	type 4	0	0	0	0	0
	Q 1030	type 5	0	0	+	+	+
	Q 454	type 6	0	0	0	0	0
	Q 902	type 7	0	0	+	+	+
	Serotype 599-52	type 8					
B	*Sh. paradysenteriae*	*Sh. flexneri*	0	+	V	0	0
	Flexner V	type 1a					
	Flexner VZ	type 1b	0	+	V	0	0
	Flexner W	type 2a	0	+	V	0	0
	Flexner WX	type 2b	0	+	V	0	0
	Flexner Z	type 3	0	+	V	0	0
	Boyd 103	type 4a	0	+	V	0	0
	Boyd 103Z	type 4b	0	+	V	0	0
	rabaulensis, rio, saigonensis	type 4c	0	0	V	0	0
		types 4d, 4e	0	+	V	0	0
	Boyd P119	type 5	0	+	V	0	0
	Boyd 88	type 6	0	+	0	0	+
	Newcastle-Manchester		0	(some G)	0	0	V
	Flexner X	variant X	0	+	V	0	0
	Flexner Y	variant Y	0	+	V	0	0
C	*Sh. paradysenteriae*	*Sh. boydii*	0	+	0	0	0
	Boyd 170	type 1	0	+	0	0	0
	Boyd P288	type 2	0	+	0	0	0
	Boyd D1	type 3	0	+	0	0	+
	Boyd P274	type 4	0	+	0	0	+
	Boyd P143	type 5	0	+	+	0	0
	Boyd D19	type 6	0	+	0	0	+
	etousae, lavington	type 7	0	+	+	0	0
	Serotype 112	type 8	0	+	0	0	0
	Serotype 1296/7	type 9	0	+	+	late	0
	Serotype 430	type 10	0	+	0	0	late
	Serotype 34	type 11	0	+	+	+	0
D	*Sh. sonnei*	*Sh. sonnei* 1	late	+	0	+	0
		(smooth)					0
	Sh. sonnei, Sh. ceylonensis A	*Sh. sonnei* 2 (rough)	late	+	0	+	
E	*Sh. alkalescens* *Sh. dispar* *Sh. ceylonensis B* *Sh. madampensis* *Sh. tiété*	Alkalescens-Dispar group	V	+	+	+	V

+ = acid or positive. 0 = negative. V = variable but never gas, except by the Manchester type.

*NOTE: Exceptions, as to biochemical actions, will be observed.

TABLE 154.—ANTIGENIC FORMULAS OF FLEXNER TYPES

TYPE	MADSON (SIMPLIFIED)	WHEELER	ADDITIONAL	
1a	I:4	I:1,2,4,5,9		
1b	I,S:4,6	I:1,2,4,5,6,9		
2a	II:3,4	II:1,3,4		
2b	II:7	II:1,7,8,9	Subgroups of type 4:	
3	III:6,7	III:1,6,7,8,9		
4a	IV:3	IV:1,3,4	Fukumi*	Lindberg†
4b	IV:6	IV:1,6	4a:IV:4	J:IV:3,4,10
			4b:IV:6	R:IV:(4),11,12
			4c:4,5,6,7,8,9	
			4d:IV:10	C:IV:3,4,(10),12
			4e:IV,10,11	
5	V:7	V:1,5,7,9		P:IV:3,4,(10),(12), (13),14
6	VI:(4)	VI:1,2,4		
X	–:7	–1,7,8,9		
Y	–3,4	–1,3,4		

*Japanese M. J. 4: 37, 1951.

†U.S.A.F. Far East M. Bull. 1: 101, 1953.

Type 3 serum: absorb type 3 serum with type 1b, 2a, and X organisms.

Type 4 serum: absorb type 4a serum with type 2a and X organisms.

Type 5 serum: absorb type 5 serum with 1a, 3, and X organisms.

Type 6 serum: absorb type 6 serum with 1a organisms.

Sh. boydii sera must be absorbed with *Sh. sonnei* 2 and Alkalescens-Dispar antigens.

Typing of Shigellae by use of polyvalent and monovalent sera has been simplified so that it may be easily performed even in small laboratories. The following points, however, must be kept in mind:*

1. Drops should not be too small.

2. The drops should contain enough bacteria that the saline control is definitely gray but not too dense. A few trials are necessary to determine the correct density. Material from solid media is definitely preferable. If for some reason broth cultures must be used, it is usually advisable to centrifuge and use the sediment for the tests.

3. It is better to use bacteria killed by heat, 100° C. for one hour.

4. Cultures which are negative when tested alive may need to be retested after half an hour's boiling because of the inhibiting, heat labile substance. Interference by this substance has been noted occasionally with *Sh. dysenteriae, Sh. ambigua, Sh. alkalescens,* and *Sh. flexneri.* In most cases the inhibition is not absolute in the sense that most of the strains which show inhibition are still slightly agglutinated after some delay.

5. The Sonne serum contains antibodies against both phases. Phase 1 (''smooth'') agglutinates strongly and promptly. Phase 2 (''rough'') agglutinates slowly and in small clumps. This is an inherent peculiarity of the phase 2 antigen and nothing can be done about it. Those who handle phase 2 of Sonne are apt to feel, quite understandably, that the serum is weak, which is definitely not the case.

6. Many individuals do not realize the advantage of using the polyvalent Flexner serum. Flexner types 1 to Y and Sonne comprise the great majority of all strains. Thus, if isolated bacteria are tested first with the polyvalent Flexner and the Sonne sera, most of the organisms can either be directly identified as Sonne, or subsequent testing may be restricted to the Lederle Flexner sera 1 to Y. Preferential use of the most prevalent sera Flexner 1, 2, 4, and 6 will further simplify the procedure.

7. Shigella antigens do occasionally occur in Enterobacteriaceae other than Shigella. Although this is a rare occurrence, it is imperative, because they do sometimes occur, to complement serologic identification by the classical cultural methods. There is little danger in issuing a preliminary report based on agglutination only, because the percentage of error

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*We are indebted to Dr. A. J. Weil of The Bronx Hospital, New York, for this personal communication concerning use of the sera.

is small. Nevertheless, the final report must be based on serologic *and* cultural identifications. Practically all of the few troublesome cases with so-called unspecific reactions or failure of the sera were due to omission of old-fashioned bacteriologic control.

8. In this respect, the test for motility is one of the most important safeguards.

9. Both as a safety precaution and as a convenience, Weil makes his slide agglutinations on the inside of the cover of Petri dishes, which he divides into squares with a glass pencil. A piece of moist, dull black cloth, placed in the bottom of the dish, gives an excellent background for readings, and provides a simple moist chamber. Readings are most conveniently made in oblique light in a dimly lighted room. For instance, Weil reads his reactions in a small cubicle used for microscopic work, using the microscope lamp tilted at an appropriate angle.

Agglutination Test With the Patient's Serum

Agglutination phenomena do not occur in bacillary dysentery until about the twelfth day from the onset. The test, therefore, is not valuable for diagnostic purposes.

Protei

Morphology: Rods, 0.5 to 1.0 by 1.0 to 3.0 microns, occurring singly, in pairs, frequently in long chains, and in threads. Very motile with peritrichous flagella. No capsules; no spores.

Stain: Gram-negative.

Biochemistry: All Protei form urease which can be determined by the urease test R-15. They ferment dextrose, sometimes also sucrose. They may or may not produce indol. All strains, except *Pr. rettgeri,* form gas, and do not attack mannitol.

Pr. vulgaris spreads over the surface of moist media at incubator temperature. *Pr. morganii* spreads only on 1% agar at room temperature. It is thought that some protei may be pathogenic for children and others. Protei X19, X2, and XK are agglutinated by the sera of patients suffering from Rickettsial infections.

Characteristics of Protei are described in Table 152.

They are easily isolated on eosin-methylene-blue media M-71 or M-72. When *Pr. vulgaris* is present in wounds, it may make isolation of other organisms difficult. Spreading of its colonies may be stopped by using sodium azide (M-21), tellurite (M-42), or phenylethyl alcohol (M-23).

Coliform Organisms

Coliform organisms include the *Escherichia coli-Aerobacter aerogenes* group and the paracolon organisms.

Both main types of coliform organisms, *Esch. coli* and *Ae. aerogenes,* ferment dextrose, mannitol, and lactose with the production of acid and gas. Many strains ferment sucrose and salicin. *Esch. coli* is more often found in warm-blooded animals than *Ae. aerogenes,* which is more often present on the surface of plants.

These organisms may be differentiated according to Table 155.

TABLE 155.—THE "IMViC" REACTION

	Indol formation R-12	Methyl red test R-13	Voges-Proskauer reaction R-14	Utilization of citrate R-17
Esch. coli	+	+	0	0
Ae. aerogenes	0	0	+	+

In addition, *Esch. coli* grows on eosin-methylene-blue agar, M-71 or M-72, in dark purple colonies, with a metallic sheen, while *Ae. aerogenes* forms large moist, white or pinkish colonies with only a dark center. *Ae. aerogenes* is closely related to Klebsiella, and is classified with it.

Organisms which show atypical reactions and cannot be classified as typical *Esch. coli* or *Ae. aerogenes* are called "intermediates."

Paracolon Organisms.—

Paracolon organisms differ from *Esch. coli* and *Ae. aerogenes* by their slow fermentation of one or more of the following substances: lactose, sucrose, salicin. They cause considerable difficulty in the diagnosis of Salmonella infections. They are classified, according to their "IMViC" reactions as *Paracolobactrum coli, Paracolobactrum aerogenes,* and *Paracolobactrum intermediate.* In addition, "anaerobic" strains are known; these form only acid but not gas from fermentable substances.

Many coliforms have Salmonella or Shigella antigens. The occurrence of such antigens does not always signify pathogenic power.

Escherichiae.—

Escherichiae have "O" (somatic), "H" (flagellar), and "K" (envelope) antigens. "L" antigens are carried only by living organisms, and can be removed from antisera produced by injecting live bacteria only by absorption with boiled cultures. These antigens are, therefore, thermolabile somatic (envelope) antigens. "L" antigens are responsible for the "O"-inagglutinability of many live strains. "A" antigens are removed by heating for 2 hours at 120° C., while "B" antigens are destroyed by heating for only 1 hour at 100° C. Antigens "L," "A," and "B" are considered subdivisions of the "K" antigen.

At the time of writing this chapter, there are 128 recognized *Esch. coli* serotypes, belonging to 26 groups. Serotypes 26, 55, 86, 111, 112, 119, 124, 125, 126, 127 and 128 have been implicated in diarrhea. They are easily identified in slide-agglutination tests using 1:10 diluted rabbit sera prepared by injecting live cultures into rabbits. Most frequently types 55 and 111 are seen.

Since *Esch. coli* 111 does not ferment sorbose but breaks salicin within 4 days, while *Esch. coli* 055 has the opposite characteristics, colonies showing the appearance of *Esch. coli* on eosin-methylene blue agar (M-71 or M-72) may be streaked to a blood agar plate. Neither of these two *Esch. coli* types causes hemolysis. The cultures are then transferred to one Kligler (M-79) or T.S.I. tube (M-80), and to broth with sorbose (M-5) and with salicin (M-5). *Esch. coli* 111 and 055 do not form hydrogen sulphide, but will produce acid and gas in the Kligler or T.S.I. tubes. The surface growth may be used for agglutination, or a separate tube with agar (M-13, M-16, or M-17) is inoculated for this purpose.

Aerobacter (Klebsiella).—

Aerobacter (Klebsiella) organisms have capsular (K), smooth somatic (O), and rough somatic (R) antigens, as well as mucoid (M) antigens. Kauffmann* suggested an antigenic schema with 4 "O" and 14 capsular types, thus 17 antigenic combinations. Many Aerobacter strains form urease.

Aerobacters frequently occur in diarrheic stools of various etiology. They have been indicated as causing intestinal disturbances in children.

Paracolobactra (Paracolons).—

Paracolobactra (Paracolons) have complicated serologic structures. Three groups, belonging in the intermediate class, are rather well defined: the *Ballerup,* the *Arizona,* and the *Bethesda* groups (see page 1526).

The *Providence* group at present is somewhat confused. Members of this group may or may not be motile; may or may not form gas; do not produce hydrogen sulphide, but are indol positive. They grow on citrate agar; do not form urease or break gelatin; and may or may not attack mannitol. They are Voges-Proskauer negative. Of the nongasforming types belonging here, Stuart's 29911 strain is the best known.

Felsenfeld finds it expedient to call "transient" those anaerogenic paracolon organisms which ferment mannitol and produce urease, as well as those which lack both abilities.

Since the issue of Paracolobactra still needs much clarification, these organisms should be considered as the cause of diarrhea only if careful examinations have excluded other possible causes, especially parasitologic.

Kauffmann and Edwards consider *Pc. coliforme* as *Esch. coli,* and *Pc. aerogenoides* as Klebsiella-Aerobacter.

Typing sera for the Paracolobactra are available only in specialized laboratories.

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*Kauffmann: Enterobactericeae, Copenhagen, 1951, Ejnar Munksgaards Forlag.

BRUCELLA

Brucella Melitensis

Morphology: Short, ovoid rods, 0.3 to 0.4 micron in length, occurring singly, in pairs, and in short chains and threads. Nonmotile, nonspore-forming, noncapsulated.

Stain: Gram-negative.

Colonies: On agar, they form ''ground glass'' appearing colonies, small, circular, convex, with dark center. They do not change litmus milk. They produce no change in the differentiating media for the colon group of organisms, since they do not ferment any carbohydrates. They are identified by their agglutination with immune sera. Cause brucellosis (Malta or undulant fever).

Brucella Suis

Morphology: Similar to *Brucella melitensis.*

Stain: Gram-negative.

Colonies: Culturally similar to *Brucella melitensis.* Isolated from genitourinary organs of swine. Chief host, the hog. Causes abortion in swine and frequently causes brucellosis (undulant fever) in man. Also infectious for horses, dogs, cows, monkeys, and laboratory animals.

Serology: *Brucella suis* can be differentiated from *Brucella melitensis,* but not from *Brucella abortus,* by the agglutinin absorption test.

Brucella Abortus
(Bacillus Abortus Bang)

Morphology: Rods, 0.4 to 0.5 micron by 0.6 to 1.5 microns, occurring singly. They are nonmotile, noncapsulated, and nonsporeforming.

Stain: Gram-negative.

Colonies: Very small, transparent, opalescent, lustrous, moist, entire. They do not change the differentiating colon bacilli media, since they do not ferment any carbohydrate. Cause contagious abortion in cattle, brucellosis in man.

Differential Characters of Species of Genus Brucella*

	INFECTIVITY FOR GUINEA PIGS	CO_2 TOLERANCE 10 PER CENT	H_2S FORMATION DAYS	DEXTROSE UTILIZED	AMINONITROGEN UTILIZED	EFFECT OF DYES	
						THIONIN	BASIC FUCHSIN
Brucella melitensis	++	0	+	+++	+	+++	+++
Brucella abortus	++	++	2	+	++	0	+++
Brucella suis	++	0	4	+++	+	+++	0

*Bergey: Manual of Determinative Bacteriology, ed. 6, 1948, Williams and Wilkins.

BRUCELLOSIS

Bruce,[1] investigating the disease known as Malta fever, Mediterranean fever, or undulant fever, discovered a bacterium in fatal cases of this disease which he called *Micrococcus melitensis.* This was a disease of goats transmissible to man. It was found on the Island of Malta, where British garrisons were often affected, and also on neighboring islands and shores of the Mediterranean. It was occasionally reported from Italy, Spain, Greece, Transcaucasia, Algeria, Tunis, Palestine, Arabia, India, South Africa, China, and various parts of South America. It was apparently brought to the United States about 1911. Bang[2] in Denmark, in 1897, isolated a bacterium responsible for contagious abortion in cattle. This disease was commonly known thereafter as Bang's disease; she termed the organism the *Brucella abortus.* MacNeal and Kerr[3] in 1910 first isolated and cultivated this organism in the United States.

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[1] Practitioner 39: 161, 1887.

It was not until the work of Evans[4] was published in 1918 that these two diseases, one of goats and man, and the other of cattle, were found to be closely related because of the remarkably similar morphologic, cultural and serologic resemblances between the two bacteria. Traum,[5] in 1914, found in fetuses prematurely expelled from sows a bacterium which is related to Bang's disease and Malta fever. The bacteria, therefore, of Malta fever, of contagious abortion in cattle, and of sows, have well been given the generic name of Brucella and are designated as *Br. melitensis, Br. abortus,* and *Br. suis.* Infection with any of these bacteria is termed brucellosis.

Laboratory Diagnosis of Brucellosis.—The best method for the diagnosis of brucellosis is the cultivation and identification of the organism. The *melitensis* and *suis* varieties of Brucella grow readily under aerobic conditions, while the *abortus* variety requires an atmosphere containing 10 per cent carbon dioxide (see Fig. 380).

Morphology and Staining Characteristics of Brucella.—Brucella are small coccal or bacillary forms varying from 0.4 to 2.2 microns in length and from 0.4 to 0.8 micron in breadth. Both coccal and bacillary forms may be present. *Br. melitensis* is generally considered to be more coccal in form than *Br. abortus* but the difference in size and shape is so slight as to render it impossible to distinguish with certainty between individual strains. The bacteria appear singly, in pairs end to end, or in small groups; sometimes short chains of 4 to 6 members may be seen, especially in liquid media. These bacteria are nonmotile.

On semisolid media, the colonies are small, circular, convex, amorphous, smooth, and translucent. No pigment is formed. The growth of *Br. melitensis* becomes brown in older cultures and the browning extends down into the medium. *Br. abortus* may show this browning at times.

Brucella may be stained with the ordinary dyes. There is a tendency toward irregular staining and in some cases bipolar staining is present. They are gram-negative, nonacid-fast, and form neither capsules nor spores. The optimum temperature for cultivation of Brucella is 37° C. No acid or gas is produced. Growth in milk is accompanied by slowly increasing alkalinity. Indol is not formed. Optimum pH is 6.6 to 6.8. All three varieties of Brucella produce hydrogen sulphide. *Br. suis* produces it in abundance, *Br. abortus* less so, and *Br. melitensis* very slightly.

Br. melitensis and *Br. suis* may be grown under the usual aerobic conditions and, after a number of transfers, the *Br. abortus* adapts itself to ordinary aerobic growth.

It is interesting to note that *Br. abortus* and *suis* may be killed in three minutes at temperatures of 140° to 145° F. They persist in soil, water, and dust for several months, but disappear within ten days in milk, presumably as a result of the acid formed by other bacteria. These bacteria are able to survive two hours or more in milk mixed with gastric juice.

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[2] Z. Thiermed. 1: 241, 1897.
[3] J. Infect. Dis. 7: 469, 1910.
[4] J. Infect. Dis. 22: 580, 1918.
[5] Rep. Chief. B. Anim. Industry, p. 30, 1914.

A Practical Method for Routine Blood Cultures in Brucellosis

A great deal of time has been lost in laboratories in attempts to justify isolation of Brucella organisms from blood. The use of large amounts of material and handling of many tubes provide sufficient opportunity for infection of technicians with Brucella strain. In order to cultivate these organisms successfully, M. Ruiz Castañeda, from the Department of Medical Research, Hospital General, Mexico, D. F., gave a practical method.[1] Dr. Castañeda's work is personally well known to the author, who considers a communication from his pen one that can be freely accepted by bacteriologists and clinical pathologists.

The medium suggested by him is as M-25.

Fifteen c.c. of the medium are placed in 100 c.c. flat-sided, rectangular bottles. The mouth of the bottle (about 1¼ cm. in diameter) is covered with thick paper and sterilized at 15 pounds for 15 minutes. The bottles are placed on their sides so that the agar sets on one of the narrow sidewalls forming an even, transparent layer. The paper is then lifted, using aseptic technic, and 10 c.c. of Castañeda broth are added. The flask is then closed with sterile rubber stoppers and the bottle again covered with the same paper cap. The air within the bottles can be replaced by suitable mixtures of CO_2 and air by any mechanical devise which avoids contamination of the double medium. This double medium should be tested for sterility before it is used. These tests are made at 37° C. for three or four days. The agar surface is wetted by the broth at 24-hour intervals.

The double medium may be inoculated with 10 c.c. of blood, the patient usually being bled with a 20 c.c. autoclave-sterilized syringe. The needle is inserted through the rubber stopper after the careful removal of the paper cap. The mixture of blood and broth is spread over the agar layer for a moment and the bottle incubated in a vertical position at 36° C. Every other day the broth is allowed to flow over the agar layer and the bottle replaced in the incubator, again in a vertical position, thus giving a series of inoculations.

Results.—When the agar layer shows colonies after 24 to 48 hours, it is likely that the culture has been contaminated. When the colonies appear 24 or 48 hours after the second inoculation, the cultivated organism has usually been found to be a Salmonella, less frequently Staphylococcus or Streptococcus, but it may be Brucella. Positive cultures of Brucella are detected more frequently after the third agar inoculation, that is, on the sixth day of cultivation. After 20 days the negative cultures may safely be discarded. The number of colonies and time of their appearance on the agar layer vary from one case to another and serve as a good indication of the intensity of the bacteriemia. Serial cultures performed twice a month have been of assistance in roughly estimating the course of the bacteriemia. During the acute phase of the disease the early appearance of innumerable colonies is the usual finding. In the chronic phase when positive, the culture shows few or sometimes a single colony in 10 or more days.

The colonies of Brucella are clearly seen through the agar layer and are easily transferred for further study. With some experience one may differentiate them from contaminants, Salmonella, and other organisms.

Castañeda's method reduces bacteriologic manipulation and material, avoids infection by technicians, and gives positive results. The rather high citrate content is intended not only to prevent clotting of the blood but also to reduce the phagocytic activity of polymorphonuclear leukocytes to a minimum. It is thought that the ease with which the Brucella colonies appear on the agar layer is due at least in part to the absence of plasma, thus reducing the bactericidal effect. This method avoids transfers and, as stated before, affords increased safety against accidental infection to the personnel.

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[1] Proc. Soc. Exper. Biol. & Med. 64: 114, 1947.

Cultivation of Brucella.—

For the excellent method of Castañeda, see page 1540.

The cultivation of Brucella is carried out also by the method recommended by Poston[6] as follows: Obtain 15 c.c. of blood from each patient by venepuncture and place in a small flask containing 4 c.c. of sterile 2.5 per cent sodium citrate solution. Inoculate each of four flasks containing 100 c.c. of liver infusion broth of pH 6.8 with 2 c.c. of the citrated blood. Incubate the flasks at 37° C., two in the room atmosphere and two in atmosphere containing 10 per cent CO_2. (See page 1575, Fig. 380, for apparatus to produce 10 per cent CO_2 atmosphere for culture of Brucella.) After four days' incubation, make daily smears of the broth cultures and stain by Gram's method. If no organisms are seen in the smears after 10 days' incubation, transplant 5 c.c. of the original cultures to 100 c.c. of liver infusion broth every three days for two weeks. Incubate original cultures and transplants for three weeks before they are reported as negative.

Definitiveness in the diagnosis of the bacteriologic research is accomplished by means of guinea pig inoculation. Poston advises inoculation of three guinea pigs with blood from each patient; two injected intraperitoneally with 2 c.c. each of citrated blood; one inoculated in the groin with 1 c.c. of citrated blood. Observe the animals daily. Beginning two months after inoculation, make tests for specific agglutinins and for cutaneous reaction to Huddleson's brucellergin at intervals of a few days. When both tests become positive sacrifice the animals. Sacrifice animals which remain negative to the agglutination test and to the skin test 4½ months after inoculation. Plant tryptose broth M-24 with the guinea pig's blood and with pieces of organs and subject to the cultural procedures described above. The cultures may then be differentiated into *abortus, suis,* or *melitensis* varieties by the agglutinin-absorption technic, the bacteriostatic action of dyes, glucose utilization and hydrogen sulphide metabolism.[7] Prepare urine, stool, bile, or spinal fluid, or other materials for cultures according to standard methods. Inoculate such material on plates of liver infusion agar containing gentian violet in concentration of 1:200,000 (M-26a). Incubate one-half of the plates aerobically for ten days; place the other half in a container having 10 per cent CO_2 atmosphere and incubate at 37° C. for three days.

The blood may be collected directly into Huddleson's tryptose broth M-24. Trypticase soy broth M-3 is also a good medium for the culture of Brucella.

Borts emphasized the necessity of making large inocula in large amounts of fluid medium and the need for frequent plating to solid media. For such plating, liver infusion agar M-26 and tryptose agar M-16 are useful. It must be kept in mind also that the plates must be incubated in a carbon-dioxide atmosphere; this is indispensable for *Br. abortus* and does not harm the other types.

Plating is made with inocula taken from the interface of the blood-broth mixture. Contamination may occur from introducing a sterile capillary pipette too often into the blood culture. To avoid this, Felsenfeld recommends use of divided cultivation of blood samples in several tubes of trypticase soy broth M-3 or tryptose broth M-24.

It is good procedure to make subcultures every seven days for three weeks.

For further differentiation, liver agar M-26 with dyes is used. Page 1538 shows the difference between the strains.

Huddleson recently has recommended the addition of dead Brucella cells to the culture media. This method yields heavier growth.

SEROLOGY

Agglutination Test for Brucella.—In the absence of positive cultures, the most reliable indicator of Brucella infection is the agglutination test. This is particularly true in cases of acute brucellosis, in which a high serum agglutinin titer will be found in most cases. There is a progressive increase in the agglutinin titer upon repeated testing in suspected cases of acute brucellosis. Agglutinins may appear as early as the fifth day of illness, but ordinarily are not found until the second week after the onset. In some instances, specific agglutinins may not appear for several weeks. It is a very important fact, which is difficult to reconcile with our immunological knowledge, that agglutinins may be persistently absent or may be present in low titer in persons from whom Brucella has been cultivated. It must also be remembered about the agglutination reaction that the titer may remain at a high level for months or years after recovery. Again, certain people exposed to the infection may develop agglutinins without any apparent illness. Next, the level of the agglutinin titer may fluctuate widely on repeated testing. It is for this reason that keen judgment is necessary in interpreting the results of the agglutination test. A person suffering from some disease other than brucellosis may have a positive agglutination test merely as the result of a previous symptomatic or asymptomatic Brucella infection.

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[6] Pub. Health Rep. 53: 1, 1938.

[7] (1) Evans, A. C.: Pub. Health Rep. 52: 295, 1937.
(2) Huddleson, I. F.: Tech. Bull. No. 100, Mich. State College, 1929.
(3) Huddleson, I. F.: Commonwealth Fund, N. Y., 1934.

In cases of chronic brucellosis, there are still more difficulties in interpreting agglutination tests. Many patients with chronic brucellosis give repeatedly negative agglutination reactions or positive tests in low titer. Attention is also to be called to the occasional cross agglutination of Brucella and *Pasteurella tularensis*. In cases of tularemia the relatively higher titer with the *P. tularensis* antigen and the usually typical history permit one easily to interpret the serologic findings. Vaccination against cholera also might give a positive titer.

The limitations of the agglutination test, as Simpson[8] so well pointed out, must be recognized; otherwise, errors will be made in two directions: first, the correct diagnosis of brucellosis may not be made because too much reliance is placed upon a negative test; or second, an incorrect diagnosis of brucellosis may be made in a person who has a residual agglutinin titer from a previous invasion by Brucella, but who is suffering from some other disease when the test is made.

Huddleson Rapid Agglutination Test for Undulant Fever of Man (Brucellosis) and Abortion Disease of Cattle

Materials Needed.—

1. Glass test tubes for collecting the blood sample. These are prepared in the manner described in the preparation of patient's serum. (See page 2031.)
2. 0.2 c.c. pipettes graduated in 0.001 c.c. These are the same as used in the Kahn test.
3. Glass plate ruled off with inch squares.
4. Huddleson antigen. This can be purchased from Sharp and Dohme, or Lederle, Inc.
5. Clean toothpicks for mixing serum and antigen.

Technic.—

Allow the patient's blood to clot. Loosen the clot from the side of the tube. Centrifuge at high speed for fifteen minutes to separate the serum from the clot. Remove the serum from the clot by means of a sterile dropper. The serum should be free of cells.

The apparatus used is a glass plate ruled off with inch squares and resting on a dark field illumination box.

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[8] Ann. Int. Med. 15: 408, 1941.

Draw up 0.2 c.c. serum in the graduated pipette.

Remove any serum from the outside of the pipette with a clean cloth. Hold the pipette vertically when measuring the blood serum.

Place 0.08 c.c. blood serum in the first square.

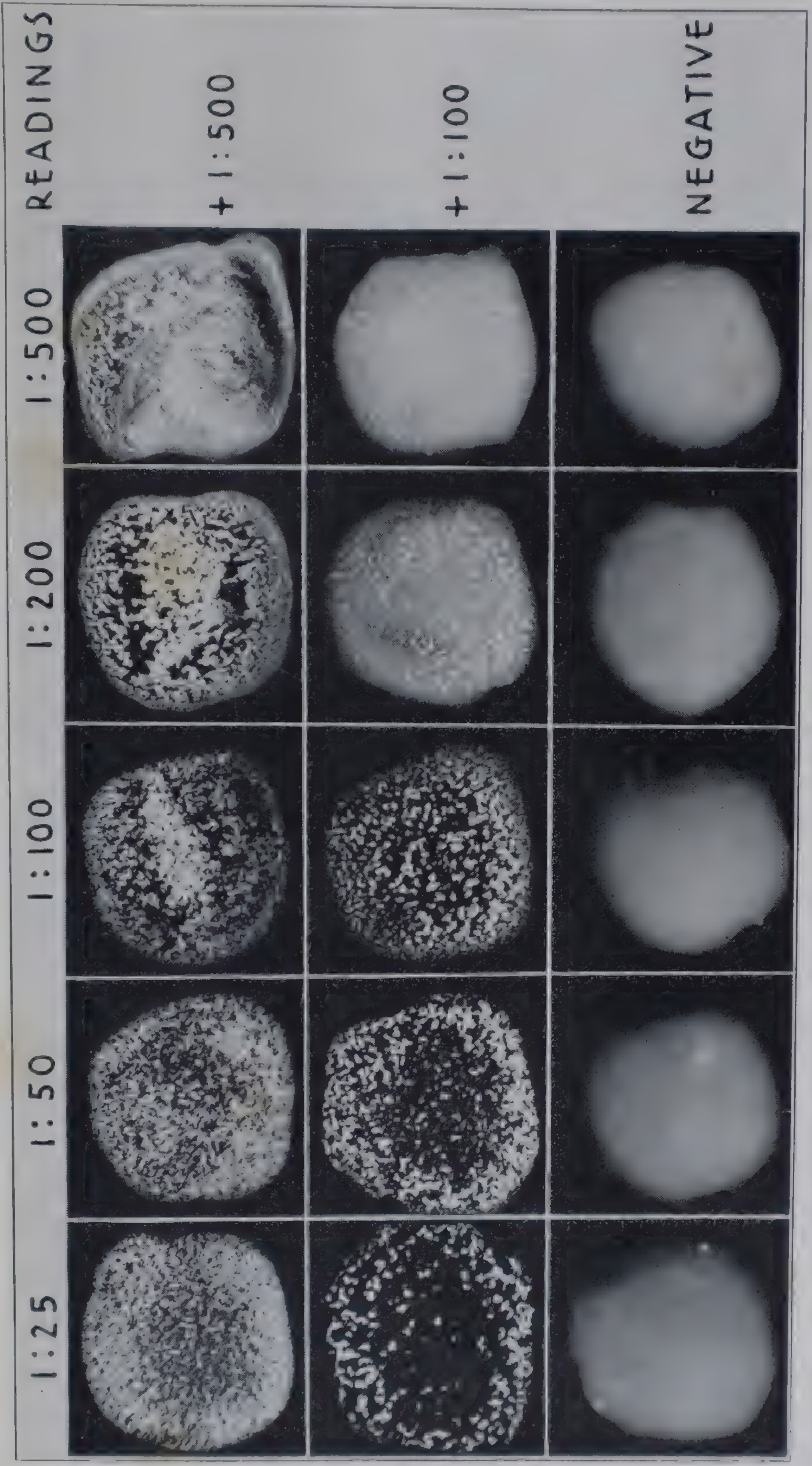

Fig. 364.—Rapid agglutination test for brucellosis. (From Gould and Huddleson: J. A. M. A., Dec. 11, 1937.)

Place 0.04 c.c. in the second square; 0.02 c.c. in the third; 0.01 c.c. in the fourth; 0.005 c.c. in the fifth.

Shake the vial of antigen thoroughly.

Remove a dropperful of the antigen. Hold the dropper vertically and add 1 drop of the antigen to each quantity of blood serum on the glass plate. Positive and negative controls should be made with the tests.

Using a clean toothpick for each quantity of serum, mix the serum and the antigen thoroughly.

Mix further by removing the glass plate from the box and tilting each back and forth 20 to 30 times. Check the degree of clumping after each 5 times. Replace the glass plate.

Examine at once for agglutination after turning on the light.

Reading the Test.—A negative result shows no change in the mixture of serum and antigen.

A trace of flocculence in the 0.08 c.c. amount of serum, with no flocculence in the other quantities, is to be disregarded, and the specimen reported as negative.

A positive reaction is reported if there is flocculence in any quantity of serum below 0.08 c.c.

Complete agglutination with the various amounts of serum used represents the following titers:

0.08 c.c.	1: 20 dilution of serum
0.04 c.c.	1: 40 dilution of serum
0.02 c.c.	1: 80 dilution of serum
0.01 c.c.	1:160 dilution of serum
0.005 c.c.	1:320 dilution of serum

With human sera, the positive reaction in a titer of 1:80 is considered diagnostic of brucellosis. With cattle sera a positive reaction in a dilution of 1:40 is suspicious of present or past infection, while positive reactions in 1:80 or higher are diagnostic.

One may expect to find the agglutinins in these cases after the blood has been infected for a week or longer.

Alternate Method of Agglutination Test for Undulant Fever of Man and Bovine Infectious Abortion

Cultivate several strains of *Brucella abortus* on liver infusion agar (M-26) for several days, wash off and suspend in 1 part carbolic acid to 200 parts normal saline solution. Make an even suspension. Filter this suspension through sterile paper and test for sterility. This sterile suspension is centrifuged and the clear supernatant fluid discarded. Pick up the sediment in a small amount of 0.4 per cent Formalin and keep the packed organisms in an ice box, making dilutions of the desired density at the time the agglutination tests are conducted.

Use unheated sera.

For each serum arrange four test tubes, adding to each one the following amounts of serum: 0.1, 0.04, 0.02 and 0.01 c.c. Add 2 c.c. of the antigen to each tube; this gives dilutions from 1:20 to 1:200. Now prepare control tube containing 0.1 c.c. of saline and 2 c.c. of antigen. Mix and incubate at 37.5° C. for twenty-four hours.

The readings are interpreted as follows:

Compare agglutination 1:50 or over implies positive result with cattle sera. With human sera, positive reaction requires agglutination at 1:100 or over.

A partial agglutination of 1:50 means a weakly positive with cattle sera.

No agglutination in 1:20 or over is negative with cattle sera.

The Brucellergin Test[9]

This intradermal test is used to determine hypersensitiveness to specific Brucella antigen. A positive allergic skin reaction is generally accepted as evidence of past or present invasion of tissues by Brucella. Most patients from whose blood Brucella has been recovered show a positive skin test, although occasionally the test is negative even though Brucella infection has been proved by culture. The chief sources of error in interpreting the significance of a positive skin test are: first, the test is frequently positive in exposed individuals with no history of an illness compatible with brucellosis; second, this hypersensitiveness, once acquired after symptomatic or subclinical infection, usually persists for many years. A positive skin test, therefore, does not mean that the symptoms from which the patient is suffering at the time of the positive skin test are necessarily due to brucellosis. Gould and Huddleson[9] believe that if the skin test is negative, brucellosis may be ruled out.

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[9] Gould, S. E., and Huddleson, I. F.: J. A. M. A. **109**: 1971, 1937.

Making the Skin Test.—A number of antigens have been used for this skin test. The most commonly employed agents are (1) a heat-killed suspension of Brucella in physiologic saline solution and (2) a suspensoid of nucleoprotein isolated from Brucella by chemical separation, known as Brucellergin (Huddleson).

Technic of the Test.—Dilute the vaccine (which is commercially available) in the proportion of one part vaccine to nine parts sterile physiologic solution of sodium chloride and inject 0.1 c.c. of the diluted suspension intracutaneously in the ventral surface of the forearm. It is necessary to use properly standardized vaccines from a reliable source.

Interpretation.—A positive reaction is characterized by the development of a circumscribed erythematous, edematous, indurated area at the site of injection. In a positive test, the area of local reaction averages about three-fourths of an inch in diameter, but may vary from one-half inch to three or more inches. The reaction reaches its greatest intensity in 24 to 48 hours. Observe the test to get the best results 48 hours after injection. The presence of mild, transient erythema, without edema and induration, is of no significance. In frankly positive cases the induration usually persists for several days. In hypersensitive persons a positive test may be accompanied by a mild, or in some instances a severe systemic reaction. In such cases, the lymphatic channels above the site of inoculation may become red, thickened and painful, and the regional axillary lymph nodes may become enlarged and tender. An exacerbation of symptoms may follow the development of a positive skin test. Focal necrosis of the Arthus type occurs at the site of inoculation in a few cases.

It is interesting to note from the observations of Goldstein[10] and Elton[11] that the injection of heat-killed Brucella for the intradermal test stimulates the production of agglutinins. Evans[12] found that the intracutaneous injection of brucellergin in twelve volunteers caused the development of opsonins in seven and agglutinins in five; in one instance the agglutinins rose from zero to a titer of 1:320. Hence, blood specimens for serologic tests should be collected before the intradermal test is performed.

The Opsonocytophagic Test for Brucella

The opsonocytophagic test is partially described on page 1573. Owing to the inadequacies of the agglutination test and the intradermal test in certain cases to distinguish between present and past Brucella infection, Huddleson, Johnson, and Hamann[13] reintroduced the opsonocytophagic reaction, a modification of the Leishman-Veitch technic for determining the phagocytic activity of the blood in the presence of serum opsonins and homologous leukocytes. This test is employed in conjunction with the intradermal test or the agglutination test, or both, to determine the immunity status of an individual giving positive tests by either or both methods.

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[10] J. Clin. Invest. 13: 209, 1934.
[11] Arch. Path. 30: 797, 1940.
[12] Pub. Health Rep. 52: 1419, 1937.
[13] Am. J. Pub. Health 23: 917, 1933.

Technic.—Mix 0.1 c.c. of the patient's citrated blood with 0.1 c.c. of a saline suspension of living Brucella which have been grown for 48 hours on liver infusion agar (M-26). The suspension should contain at least six billion organisms per c.c. of physiologic saline solution of a pH of 7.0. Incubate the mixture at 37° C. for 30 minutes, after which remove a small amount of the sedimented cells with a capillary pipette. Make a smear from a large drop of the cell suspension, dry rapidly, and stain with any good blood stain. The number of bacteria in 25 segmented neutrophilic leukocytes is classified according to the number of bacteria per neutrophile; the absence of phagocytosis indicates a negative result; 1 to 20 phagocytized bacteria indicates slight phagocytosis, 21 to 40 moderate, and 41 or more, marked phagocytosis.

Huddleson interprets the results as follows: The opsonocytophagic power of the blood is low during the active infective phase of the disease and becomes marked after recovery. On this basis, individuals have developed immunity to Brucella if 60% or more of the segmented neutrophiles show marked phagocytosis. If as many as 40% of the leukocytes show moderate to marked phagocytosis, the patient may be infected and has not yet developed any immunity, or he may be uninfected. According to Simpson,[8] while the contentions of Huddleson and his associates are strong in estimating the value of this test, it lacks confirmation. Studies by Calder[14] and Keller, Pharris and Gaub[15] provide support to Huddleson's thesis. On the other hand, Evans[16] regards the opsonocytophagic test as the least reliable of the diagnostic tests in cases of chronic brucellosis; she found strongly positive or immune reactions in four cases from which Brucella were cultivated, and weak or moderate reactions in recovered cases. Morales Otero and Gonzalez[17] found no correlation between cutaneous allergy to Brucella and the opsonocytophagic reaction. Lee Foshay[18] and Simpson,[8] working independently, have found that a significant proportion of patients yields aberrant and unexpected results in relation to the immunity status when the opsonocytophagic test is employed in conjunction with cultural methods, agglutination tests, and skin tests. High phagocytic titers or immune reactions occur in some patients with severe and uninterrupted brucellosis, proved by cultures. Certain recovered patients, asymptomatic for months or years, exhibit marked fluctuations from month to month, running the entire gamut from high to low phagocytosis, or sometimes none at all. Therefore, the results of the opsonocytophagic test should be interpreted with caution and with reservations.

Tovar[19] pointed out that it is necessary to use sodium citrate in a final concentration of 1% in order to eliminate "normal" opsonins. He recommended the use of formol-killed Brucellae for this test.

Testing Milk for Brucellosis.—See pages 1677 to 1680.

Hematology of Brucellosis

Leukopenia occurs in patients with acute brucellosis. In chronic brucellosis, either leukopenia, moderate leukocytosis, or normal leukocyte levels may be found. Lymphocytosis is a striking and constant feature of the blood picture in all of the manifestations of brucellosis. This is true in both percentage values and in absolute numbers of lymphocytes and there is unusually high proportion of immature lymphocytes. Mild anemia of the macrocytic normochromic type is present. The red cell sedimentation rate is usually not high, except when regional complications are present. Calder[20] believes that this combination of white blood cell and red blood cell abnormalities is distinctive for brucellosis and provides an additional confirmatory diagnostic aid.

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[14] South M. J. 32: 451, 1939.
[15] J. A. M. A. 107: 1369, 1936.
[16] Evans, A. C.: loc. cit.
[17] Proc. Soc. Exper. Biol. & Med. 40: 100, 1939.
[18] Am. J. Clin. Path. 10: 176, 1940.
[19] J. Immunol. 49: 203, 1944.

BACILLI

Bacillus Anthracis
(Anthrax Bacillus)

Morphology: Rods, 1.0 to 1.25 by 5 to 10 microns long, with square to concave ends, occurring in long chains. Nonmotile. Spores central, 0.7 to 0.8 by 1.5 to 1.75 microns.

Stain: Gram-positive.

Colonies: Irregular, fringed, ''Medusa head,'' made up of interlacing chains which lie parallel along the margin. They produce a grayish, tough, spreading, fimbriate growth on agar. They form acid in dextrose. They are aerobic. Cause anthrax (malignant pustule). Flat, translucent, grayish colonies, showing granular surface with filaments (''medusa head''—visible often only under magnification) are characteristic. Capsulated forms are found only in tissues.

Stein[21] summarized the characteristics of *B. anthracis* as: (a) causes fatal septicemia in guinea pigs, (b) nonmotile, (c) methylene blue not reduced in 48 hours, (d) salicin not fermented (M-5), (e) slow action upon gelatin (M-84), (f) slow peptonization of milk (M-11), (g) broth (M-2) is clear or shows slight turbidity, (h) sheep blood cells (R-3) are only exceptionally and very slightly hemolyzed, (i) on nutrient agar (M-13 to M-15) growth shows tenacious texture, comes off in threads when touched with the needle.

For the isolation of *B. anthracis* use nutrient agar plates (M-13 to M-15).

It is recommended that poured plates be prepared. The deep colonies of *B. anthracis* are loose and ragged, which permits differentiation from the colonies of several other of the Bacillus species. Surface colonies in transmitted light often have a ''ground-glass'' appearance.

The excretions and secretions of infected animals and man are examined. In man, the stools, urine, and pus from pustules are usually selected for the test, although the organisms may be found in the skin, intestines, and lungs. Since bacteriemia is a common complication of anthrax infection, the blood is the material of choice.

In animals, the modus of infection is considered in the selection of material. In living animals, the hair, food, and soil on which they graze are important. In dead animals, the cadaver is examined for evidence of infection, the inner organs being selected for cultures. In advanced decomposition of animals, where cultures would be almost impossible, the precipitin method of Ascoli is used. This method is a mixing of immunized rabbit serum with an extract of organs under examination for the production of a precipitate.

Ascoli Precipitin Test

Grind up the organs or blood of suspected animals.

Suspend in saline.

Boil for 5 minutes.

Filter through filter paper and allow to cool.

Carefully stratify 0.5 c.c. of this extract on 0.5 c.c. of immune serum in a small test tube.

Allow to stand 15 minutes at room temperature.

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[20] J. A. M. A. 112: 1893, 1939.

[21] Am. J. Vet. Research 5: 38, 1944.

Definite ring-like precipitate forms at the junction of the two fluids in positive cases.

Always run controls with (1) known anthrax extract; and (2) with a normal serum.

The examination of blood of infected animals will usually show typical anthrax bacilli even in a stained smear. Cultures on dextrose agar may be made for definite diagnosis.

Bacillus Subtilis

(Hay Bacillus)

Morphology: Rods, 0.365 by 1.5 to 2.5 microns, occurring singly and in chains. Motile by means of peritrichous flagella. Spores central or excentric, 0.875 micron in thickness.

Stain: Gram-positive.

Colonies: Spreading, grayish, ameboid, with crenate margin. They grow abundantly on any medium, producing a thin, grayish white membranous, glistening, spreading, adherent growth. They alkalinize and peptonize milk. They form acid, but no gas, in dextrose and sucrose. They are aerobic. Nonpathogenic. This is a frequent laboratory contaminating organism.

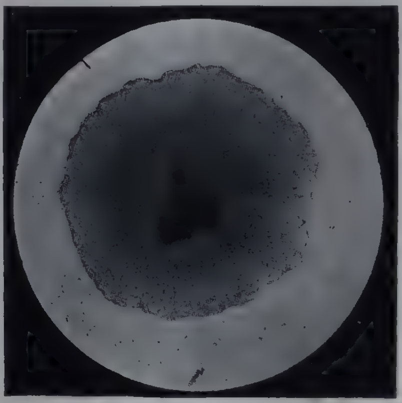

Fig. 365.

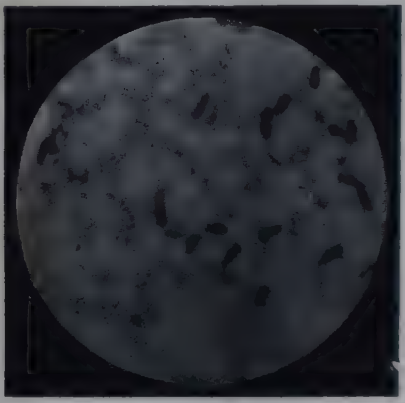

Fig. 366.

Fig. 365.—Colony of *Bacillus subtilis* (hay bacillus) on dextrose agar.
Fig. 366.—Spores of *Bacillus subtilis.* (×950.)

Bacillus Megatherium

Morphology: Rods, 1.6 to 2.0 by 3.5 to 4.0 microns, occurring singly, in pairs, and chains. Motile by means of peritrichous flagella. Spores are central or slightly excentric, and are 0.75 to 1.25 by 1.5 to 2.0 microns.

Stain: Gram-positive.

Colonies: Circular, thick, white to cream color, entire. They produce a dirty white, smooth, glistening, slimy growth on agar, browning the medium. Old cultures become yellowish red with pellucid spots over the surface. They do not coagulate milk. They peptonize the milk. They form acid in dextrose and sucrose. They are aerobic. Nonpathogenic.

Bacillus Cereus (Mycoides or Anthracoides)

Morphology: Rods, 1.3 to 1.4 by 3.0 to 5.0 microns, occurring in long chains. They are motile by means of peritrichous flagella. The spores are central, and are 0.8 by 1.4 to 2.2 microns.

Stain: Gram-positive.

Colonies: Grayish, spreading, rhizoid. They grow on agar with a whitish, soft, glistening, rhizoid growth, the growth becoming dull. They do not coagulate milk, but peptonize it slowly. They form acid in dextrose and sucrose. They are aerobic. Nonpathogenic.

GENUS CLOSTRIDIUM*

The salient features of this genus include the following characteristics:

1. Gram-positive in young cultures.
2. Catalase negative.

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*See footnote, p. 1554.

3. Sporeforming bacilli, filamentous and plectridial forms often occurring.

4. No demonstrable cytochrome system.

5. Motile, with the exceptions of *Cl. perfringens, Cl. filiforme,* occasional unclassified microaerophilic strains, and some nonproteolytic, nonsaccharolytic types.

6. Nonencapsulated, except *Cl. perfringens, C. butyricum,* and *Cl. fallax.*

7. Anaerobic, with the exception of the listed microaerophilic types: *Cl. tertium, Cl. carnis,* and *Cl. histolyticum.* Additional unclassified microaerophilic strains occur in soil and have been isolated from wounds.

The classification presented here, like that of Reed and Orr, is primarily based upon the reaction obtained in iron-milk. Four categories are evident, although, properly speaking, stormy fermentation differs from a condition of "acid, clotting, with gas" only in time and intensity.

Clostridia are predominantly saccharolytic, proteolytic, or neither. Pathogenicity is not limited to any one of these three divisions, nor is it excluded from any.

The size of the Clostridium is not a diagnostic feature inasmuch as various media alter morphology by promotion or depression of growth.

The location of the spores should not be heavily relied upon as a strong differential characteristic in presumptive identification.

The isolation and identification of Clostridia demand that critical media be employed and that they be evaluated within a reasonable time limit. Forty-eight hours' incubation at 37° C. is considered optimal for most physiologic reactions, and the present key is prepared on this basis.

Clostridium Tetani
(Tetanus Bacillus)

Morphology: Rods, 0.4 to 0.6 by 4.0 to 8.0 microns, occurring singly. They are rapidly motile by means of peritrichous flagella. Spores are spherical and terminal.

Stain: Gram-positive.

Colonies: Colonies are small, transparent, villous to fimbriate margins.

Cl. tetani is one of the strictest of the anaerobes, and is most notable for the powerful exotoxin produced. A characteristic type of death is observable in a test animal: an opisthonic convulsion is diagnostic.

It is still questionable whether there is any proliferation of the organisms in tissue, but a toxin, lethal to man in a dosage of 0.25 mg., is produced; this causes tetanus or lockjaw.

Physiologically, *Cl. tetani* is nonsaccharolytic and is a proteolytic species. Reports vary on two rather important criteria for physiologic differentiation: iron-milk and the production of indol. Normally (this may be a geographic difference), no change occurs in iron-milk (M-5), although rarely a strain will be encountered which will form a soft clot. Indol might not be produced, particularly with fresh isolations (R-12).

The Gas Gangrene Group

With each new war wound study, another evaluation of the Clostridia of the gas gangrene group is usually advanced. Members incriminated in this infection are derived from all four categories of our differential charts. It has been usual to consider as causative agents those pathogens isolated from gangrenous infections, using the term "contaminating or concomitant species'' for the nonpathogenic species which have been isolated. It is not clear what role some of the nonpathogenic proteolytic species play, but their appearance often seems to indicate more than saprophytic opportunism.

Three groups of Clostridia can be outlined which will comprise most Clostridia isolated from war wounds or cases of gangrene:

I. Proteolytic Clostridia which include *Cl. novyi* and *Cl. sporogenes, Cl. sordelli,* and *Cl. bifermentans, Cl. histolyticum,* and *Cl. lentoputrescens.*

II. Saccharolytic types of pathogenic Clostridia which include *Cl. perfringens, Cl. carnis, Cl. fallax, Cl. septicum, Cl. feseri,* and perhaps *Cl. aerofoetidum,* although the last mentioned is but slightly, if at all, toxigenic.

III. Other Clostridia often found are:

(a) Those species probably representing fecal contamination, such as *Cl. tetanomorphum, Cl. cochlearium,* etc.

(b) Those species which probably are soil contaminants: *Cl. paraputrificum, Cl. multifermentans, Cl. capitovale,* etc.

(c) In the Far East, the Armed Forces Laboratories have consistently found ''unclassified'' microaerophilic types in many wounds and have confirmed their identity from soil samples taken from the immediate area of wounding. These types are usually of slight toxicity.

Clostridium Perfringens (Welchii)
(Bacillus Aerogenes Capsulatus)

(Gas Bacillus)

Morphology: Rods, short, thick, 1.0 to 1.5 by 4.0 to 8.0 microns, occurring singly and in pairs, less frequently in short chains. They are nonmotile. They form capsules. Spores are oval, central, or excentric.

Stain: Gram-positive.

Colonies: They are obligate anaerobes. They form acid in litmus milk, coagulating it with profuse gas formation. Colonies are circular, moist, slightly raised, opaque center, entire.

Several types are differentiated according to their toxins; one of them (F) is nonhemolytic, and is supposed to cause food poisoning.

Clostridium Sporogenes (Bacillus Oedematis Maligni)

Morphology: Rods, occurring principally in long chains. They are large with rounded ends. They are motile by means of peritrichous flagella. Spores are oval and excentric.

Stain: Gram-positive.

Colonies: They are obligate anaerobes. Colonies are delicate, spreading, semitransparent, becoming grayish, crenated. They acidify and coagulate milk with gas formation.

Clostridium Feseri (Chauvei)

Morphology: Rods, large, occurring singly and in short chains. Usually show a dark chromatic point near each extremity. Motile by means of peritrichous flagella. Spores are oval, central, or excentric.

Stain: Gram-positive.

Colonies: They are obligate anaerobes. Colonies are small, grayish, semiopaque, filamentous. Cause symptomatic anthrax (blackleg).

Clostridium Botulinum

Morphology: Rods, large, with rounded ends, occurring singly and occasionally in short chains. They are motile by means of peritrichous flagella. Spores are oval and subterminal.

Stain: Gram-positive.

Colonies: They are obligate anaerobes. They grow only at temperatures below body temperature. Colonies are flat, grayish, irregular, mottled. They form an exotoxin which is pathogenic. The organisms, themselves, are nonpathogenic.

Cl. botulinum, by type being synonymous with *Cl. parabotulinum,* occurs in soil, and has been isolated from the feces of animals. Occurrence of *Cl. botulinum* seemingly tends toward a definite geographic distribution. As a point of interest, although several descriptions of cases of suspected *Cl. botulinum* intoxication exist in Japanese literature, it was not until 1950 that the exact identity of *Cl. botulinum,* type A, was undeniably confirmed from soil isolates. Type E, however, also occurs.

It is difficult to correctly identify the *Cl. botulinum* by physiologic reactions and by morphology in the absence of toxin neutralization or protection testing. On primary isolation, the organisms frequently are slow carbohydrate fermenters. The inherent pattern of physiologic reactivity, apart from the toxin, is that of the "acid-digestion" group of proteolytic Clostridia.

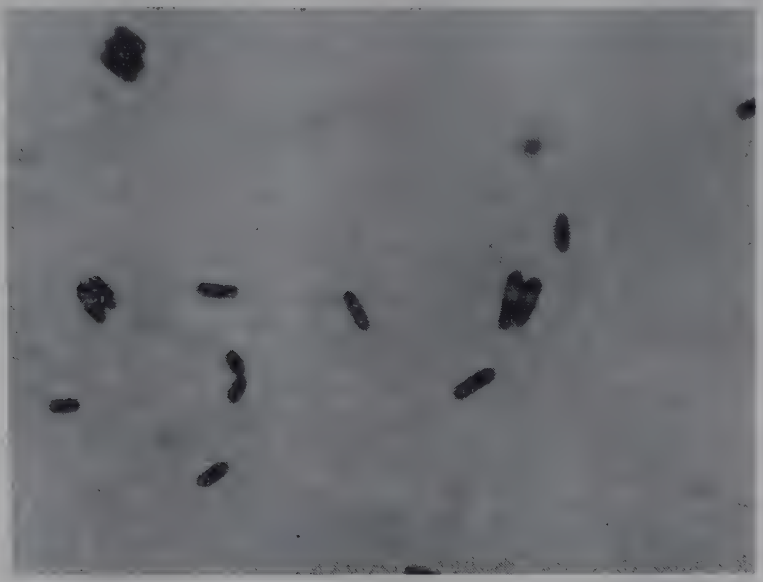

Fig. 367.—Impression smear from experimental gas gangrene, showing sporulating organism, *Clostriduim histolyticum.* (Photomicrograph from slide of T. F. Wetzler.)

Cl. botulinum is a saprophyte and probably does not proliferate in the tissue of the host.

The toxin is lethal to man in a dosage of 0.0084 mg. (crystallized).

Cl. botulinum, as a group, is divisible into some 5 types by serologic-toxigenic differentiation. Types A and B are those found in food poisoning of man; type C (often further subdivided into "alpha" and "beta") is incriminated in forage poisoning of horses, limberneck of chickens, etc. Type D has been the causative agent of a type of cattle poisoning, whereas type E may be analogous to type C.

The Butyricum Group

Since this group is useful in commercial enterprises, the manufacture of solvents and dairy products, etc., it has been well studied as a group, the notable feature of many members being "stormy fermentation" in iron-milk. Frequently, members of this group give delayed types of reactions, thus being easily separated from *Cl. perfringens.*

It has been pointed out elsewhere that the reaction termed "stormy fermentation" is a condition of "acid, clotting with gas." Fermentation of carbohydrates demonstrates the close relationships of the two categories, both of which include most of the saccharolytic types of Clostridia described.

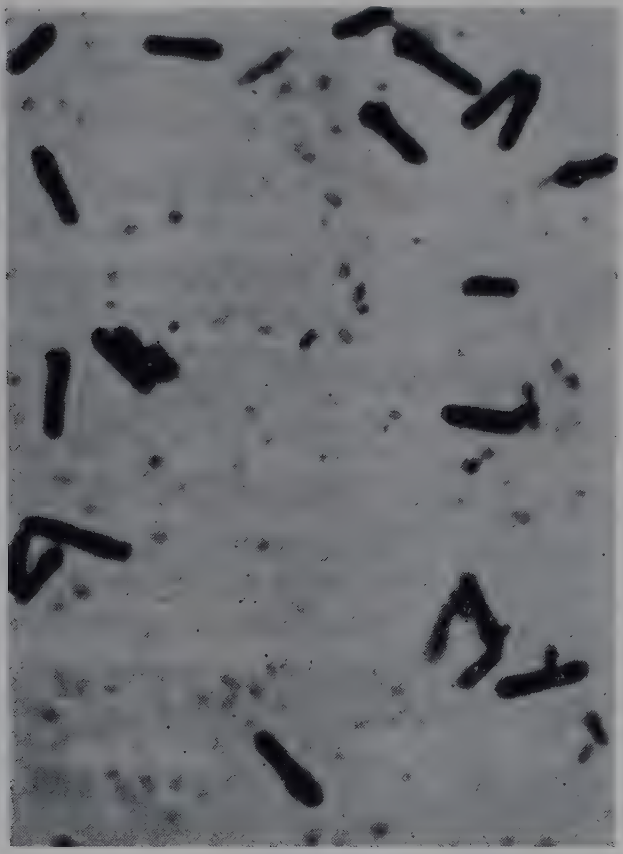

Fig. 368.

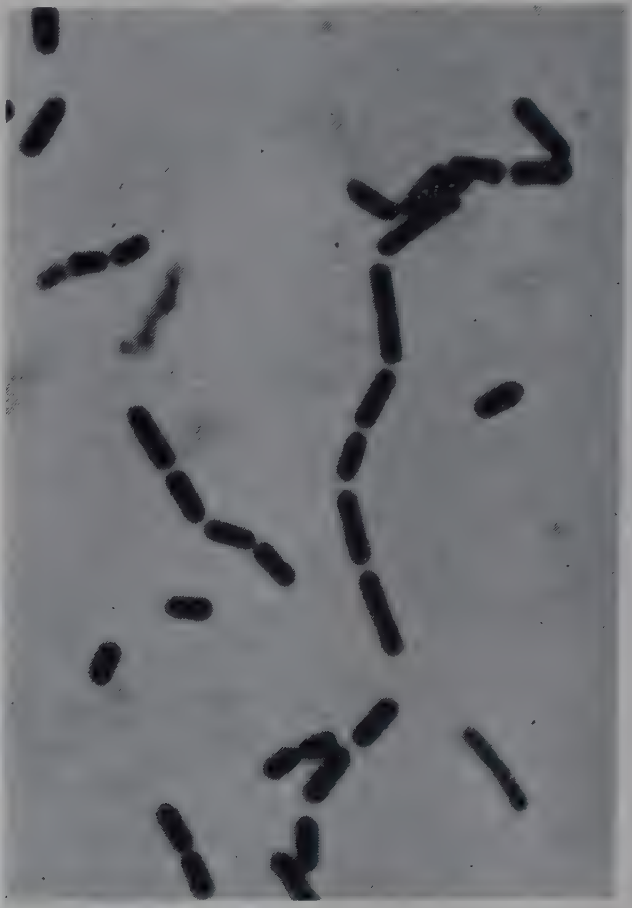

Fig. 369.

Fig. 368.—An 18-hour culture of a proteolytic Clostridium with early sporulating forms. *Clostridium sporogenes.* (Photomicrograph from slide of T. F. Wetzler.)

Fig. 369.—A typical 18-hour culture of *Clostridium perfringens*, demonstrating uniform cell size and a tendency to form short chains. (Photomicrograph from slide of T. F. Wetzler.)

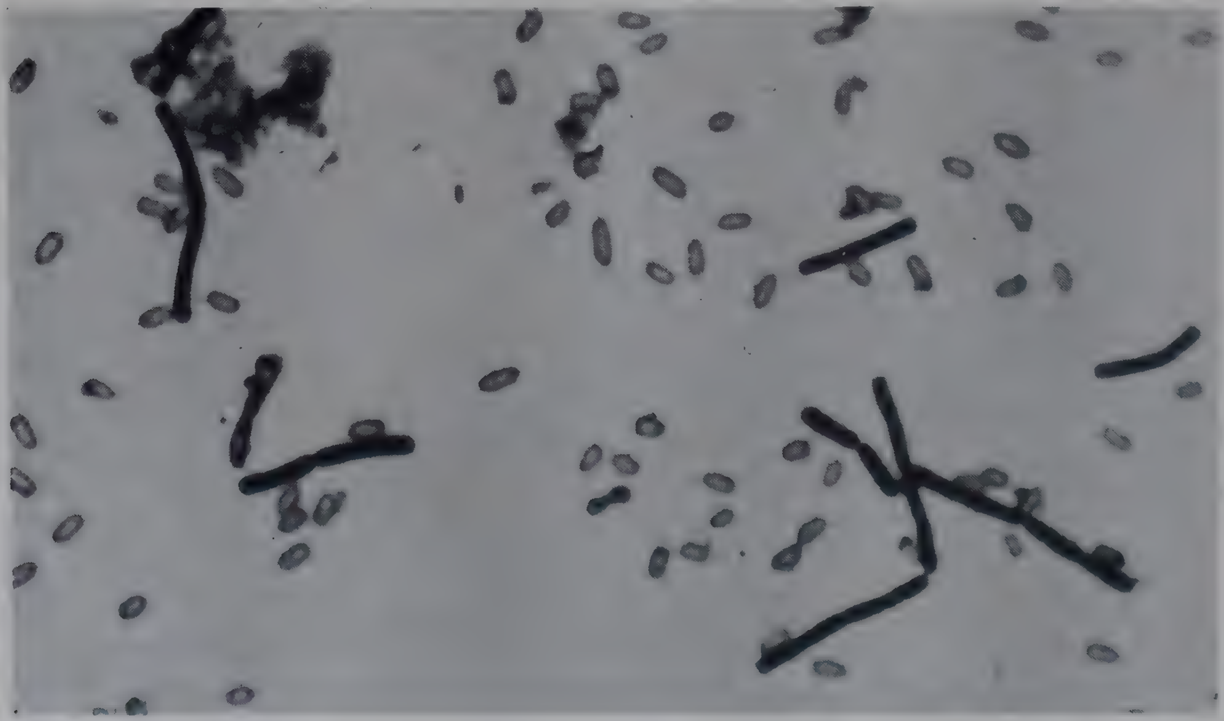

Fig. 370.—A 48-hour culture of a sporulating anaerobe. Note the relative abundance of extracellular spores. *Clostridium bifermentans.* (Photomicrograph from slide of T. F. Wetzler.)

Though it may be convenient to group all the members into a general category "*Clostridium butyricum* group," this is a haphazard approach. Many members of this group are well described: *Cl. pasteurianum, Cl. multifermentans, Cl. beijerinckii, Cl. acetobutylicum,* among others.

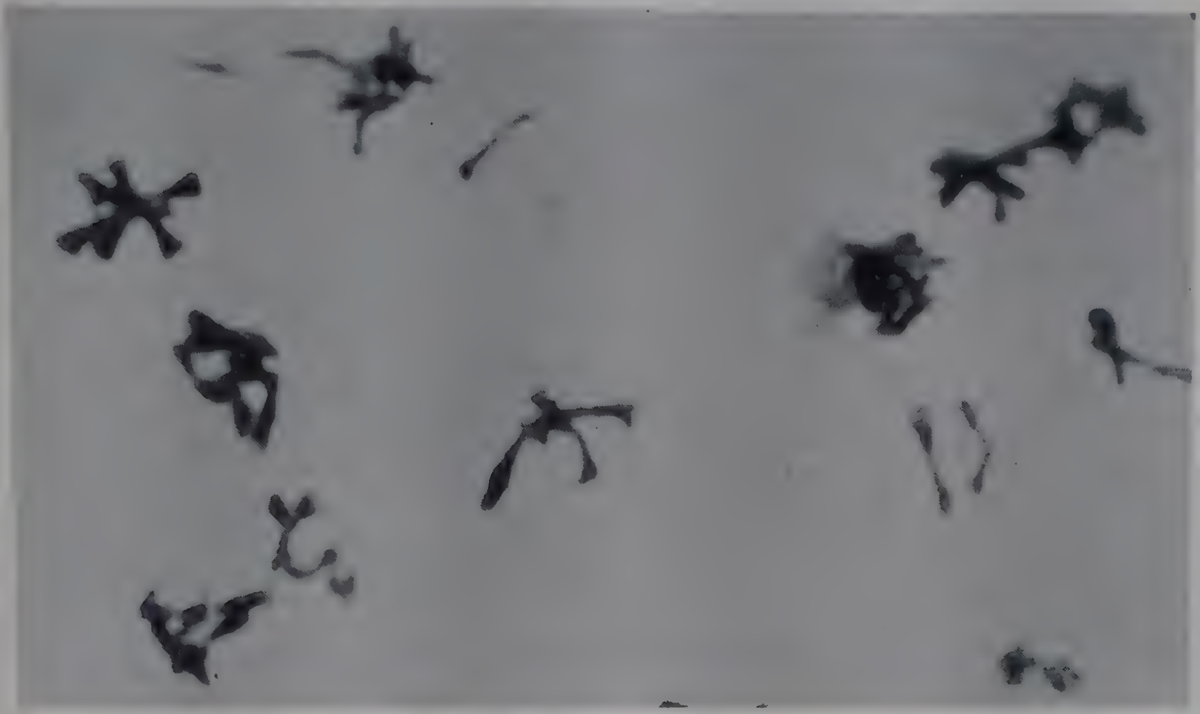

Fig. 371.—An example of pleomorphism and involution forms. A microaerophilic Clostridium. (Photomicrograph from slide of T. F. Wetzler.)

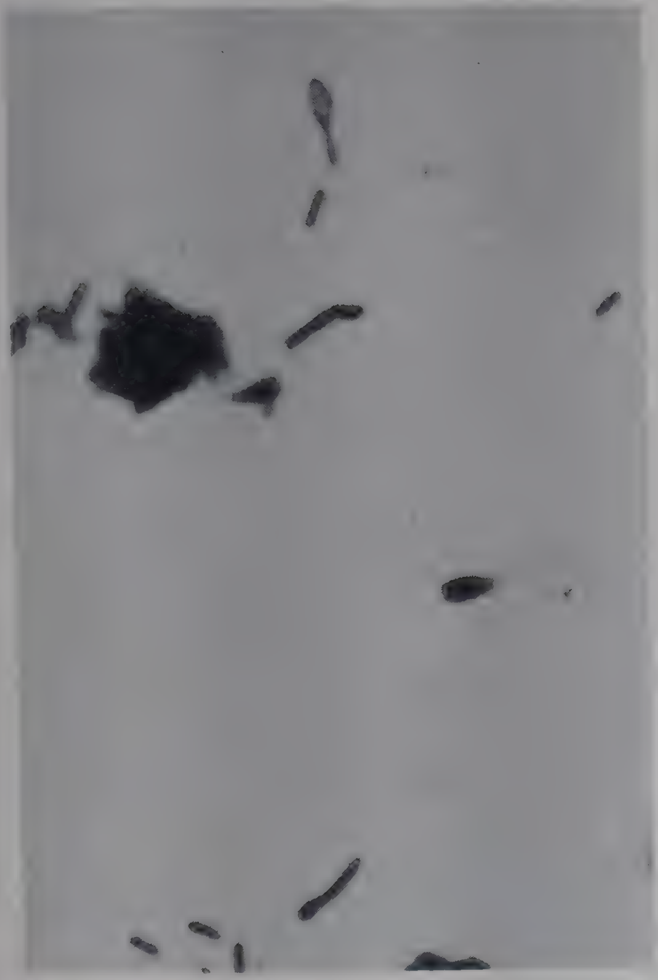

Fig. 372.

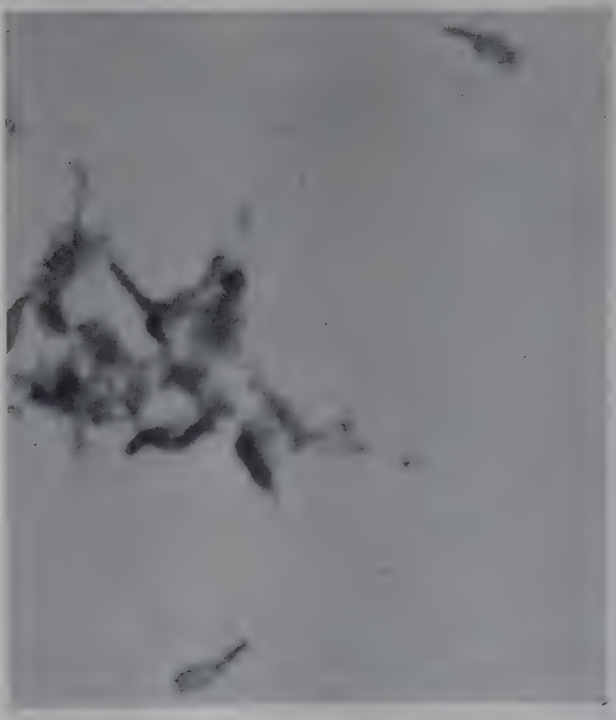

Fig. 373.

Fig. 372.—A typical microaerophilic Clostridium. Spores are not formed aerobically. (Photomicrograph from slide of T. F. Wetzler.)

Fig. 373.—Typical swollen, elongate, terminal spores of a microaerophilic Clostridium. (Photomicrograph from slide of T. F. Wetzler.)

With the exception of *Cl. multifermentans,* none of the species in this group are hemolytic.

For practical purposes, differentiation is based largely upon specific carbohydrate fermentation, the final acidity achieved being lower than pH 5.2.

There is no more difficult task for the bacteriologist than to differentiate Clostridia, particularly if they occur in mixed cultures. Much progress was made possible by the introduction of thioglycollate media by Brewer and the great contributions of McClung, Reed and Orr, and others in this field.

CLOSTRIDIA*

Introduction

Diagnostic identification of the clostridial flora always involves a three-step procedure: elimination of the concomitant aerobic flora, separation of the individual anaerobic species, and identification of the anaerobic species.

It is well to keep in mind that the members of this genus are notable for their proteolytic or saccharolytic activity or both. Reagents and media must be uniformly prepared—indiscriminate substitution frequently results in observations differing from those described.

There is no substitute for repetitious plating and exact following of procedure. There are virtually no easy tests in this field of bacteriology.

If a laboratory is not equipped to employ the various methods described for differential diagnosis, it is recommended that the clostridial isolates be forwarded to a laboratory which is prepared to use these methods.

Collection of Specimens

The source of the specimen will determine the method of collection and mode of shipment. Specimens will usually come under one or more of the following headings:

1. Material from animal sources, such as tissues from biopsy and autopsy, swabs, blood specimens, aspirated fluids, urine, feces, gastric contents, sputum, fragments of skin, fur, hair, or feathers.

Such materials are inoculated into screw-capped tubes containing chopped heart medium (M-12), or, if not available, into thioglycollate broth tubes (M-48), containing at least 20 c.c. of the medium in 50 c.c. tubes.

Specimens, particularly tissue blocks, may be shipped in the frozen state.

About 1 gm. of the following specimens is required:

2. Food specimens including raw, pasteurized, processed, or frozen materials.

Canned and pasteurized products should be submitted in original containers, regardless of the size of the container.

Frozen foods should be shipped in the frozen state.

Raw foods may be forwarded in sterile, dry containers, or, if possible, in thioglycollate broth tubes (M-48).

Samples from opened containers may be submitted for anaerobic investigation in deep thioglycollate broth tubes (M-48). One gram is a sufficiently large specimen.

Sterile, dry, screw-capped tubes are the containers of choice for:

3. Soil samples.

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*This method has been described in the *Annual Reports*, Technical Section, of the 406 Medical General Laboratory of the U. S. Armed Forces in Tokyo, Japan. We are indebted to Captain John D. Marshall, Jr., MSC, from the 406 Medical General Laboratory, and to Mr. Theodore Wetzler, now at the Vestal Laboratories, St. Louis, Mo., for details, tabulations, and photomicrographs appearing in this section.

4. Clothing specimens. A fragment approximately 1 by 2 inches is desirable.

5. Material enclosed in tissue is usually considered analogous to a tissue specimen, and is handled as such. Such materials include cloth, shell fragments, soil, etc.

6. Drugs, solvents, diluents, antibiotics, chemical preparations, etc., should be submitted in their original containers, if practicable.

Procedure

A. Preliminary Processing.—

At least one medium must retain the entire bacterial flora until all the species have been isolated and identified. The loss of a bacterial population due to faulty technic, lack of anaerobiosis, overuse of selective inhibitors, etc., occurs frequently.

I. Specimens arriving in chopped heart medium or thioglycollate broth are immediately incubated and kept at 37° C. for 24 hours. Blood specimens are incubated for 5 or more days.

II. Specimens mailed without specific media are handled in the following manner:

1. Blood specimens are thoroughly mixed by inverting several times. A 5 c.c. quantity is aseptically withdrawn and inoculated into a chopped heart medium tube (M-12) and incubated at 37° C. for 7 to 10 days.

2. Food specimens are aseptically opened. A 1 gm. quantity is inoculated into a thioglycollate broth tube (M-48).

3. Drugs, solvents, diluents, antibiotics, chemical preparations, etc., are aseptically pipetted into chopped heart medium (M-12), using 2 to 4 c.c. Solids or dry specimens may be directly inoculated into chopped heart medium.

III.

1. After this initial incubation from all tubes, 1.0 to 1.5 c.c. are aseptically withdrawn with a pipette (after thorough shaking) and inoculated into thioglycollate broth tubes (M-48) containing 6 c.c. of broth.

2. The thioglycollate broth subcultures are incubated at 37° C. for 24 hours.

3. The original tubes are kept at room temperature serving as holding media.

B. Separation of the Aerobic and Anaerobic Flora.—

1. After the incubation of the thioglycollate subculture, a Gram stain is made. Any method is acceptable, using absolute alcohol or 95% alcohol alone for decolorization. A careful check for gram-positive bacilli, particularly for those with spores, is made. Note relative position of any spores and swelling.

2. Prepare an acrolein test (R-19).

Magenta indicates positive, pink to rose is doubtful, yellow or colorless is negative.

The production of acrolein (acrylic aldehyde) from glycerol is considered specific for the aerobic *Bacillus amaracrylus,* and for the anaerobic *Clostridium perfringens.* Doubtful readings may occur with strains of *Cl. tetani, Cl. botulinum,* and *Cl. sporogenes.*

3. Inoculate solid media from tube B (1) for aerobic and anaerobic growth: (See also "Wound Infection," page 1603.)

(a) Aerobic
    (1) Bood agar with yeast extract (M-14)
    (2) Eosin-methylene blue agar (M-71 or M-72)
    (3) Tellurite blood agar (M-41 or M-42)
(b) Anaerobic
    (1) Blood agar with yeast extract (M-14)
    (2) Blood agar with sodium azide and chloral hydrate (M-22)
    (3) Nagler plate (M-57)
(c) Incubate the aerobic plates at 37° C. for 24 hours.
(d) Incubate the anaerobic plates at 37° C. for at least 40 hours, but no longer than 60 hours.
(e) Anaerobiosis is best accomplished in the Brewer anaerobic jar under positive hydrogen pressure, which is supplied by using mossy zinc and 30% hydrochloric acid in a generator. Powdered chromium and sulphuric acid may be used, too. Illuminating gas can be substituted for hydrogen, as in the Gradwohl-Drucker jar.

(f) Indicators of anaerobiosis must be employed. A glucose-methylene blue tube is preferred for this purpose.

4. Growth on the aerobic plates is noted and recorded.

5. The anaerobic plates are examined after 40 to 60 hours' incubation and processed as follows:

(a) Check the anaerobic controls, dismantle the jar, and remove the plates.

(b) Observe colony formation.

No test adequately describes colostridial colony morphology on the plates. Competence is acquired only through practice and familiarity with the media employed. Before attempting to pick colonies, compare the yeast extract blood agar plate with the sodium azide-chloral hydrate plate. Most mesophilic bacteria grow as well as Clostridia on the anaerobic plates, but Staphylococci, Bacilli, and most Enterobacteriaceae do not grow on the sodium azide-chloral hydrate medium.

The Gram stain will easily demonstrate the inhibition effected.

All gram-positive spore-bearing bacilli which do not grow well on aerobic plates are suspected of being Clostridia.

In general, the following colonies are picked:

All hemolytic colonies
All transparent colonies
All flat, grayish, translucent colonies
All colonies with emanating dryish spreading growth.

6. The colonies are picked and transferred into thioglycollate broth (M-48), incubated overnight at 37° C., and the growth examined with the aid of slides, stained according to Gram.

7. All gram-positive bacilli, regardless of sporulation pattern, are streaked out on yeast extract blood agar plates (M-14), and incubated for 48 hours. Should minute colonies arise within this time, not to exceed 1 mm. in diameter, a Gram stain must be made on them. Gram-positive bacilli showing such colony formation are probably microaerophilic Clostridia. It has been reported that microaerophilic Clostridia do not sporulate on aerobic plates.

8. Any questionable findings encountered require that the thioglycollate broth culture be replated anaerobically and colonies picked anew.

9. All findings are to be duly entered on the work sheets.

10. One of the more difficult points to be emphasized in anaerobic work is the necessity for constant checking of an isolated strain for contamination. The isolation of but a single species of Clostridium from an incoming specimen is a rare occurrence. As a rule, when one species is isolated, it is the practice to repeat the isolation technic to find others. If Clostridia are recovered from a specimen, at least 5, and usually 10, pickings of each should be employed for taxonomic study. If no growth is observed, the initial tube should be subcultured again, in order to avoid false negative results.

Taxonomic Classification of Clostridia

A. Introduction.—

Clostridia may be roughly classified into three general patterns, none of which is mutually exclusive: pathogenic and nonpathogenic, toxogenic and nontoxogenic, proteolytic and saccharolytic.

The needs of clinical bacteriology are usually met by the rough division into pathogenic and nonpathogenic types. Pathogenicity varies with the type of laboratory animal used. For uniform results, the guinea pig is the animal of choice.

A table listing differential characteristics is included. Geographic distribution of clostridial species may result in occasional variations, perhaps in carbohydrate fermentation, liquefaction of gelatin, or in the rapidity of hydrogen sulphide production.

B. Differential Criteria.—

Differential criteria are shown in Tables 156 to 159, which follow mostly that of Reed and Orr.

1. All differential media used in this work are to be inoculated with 0.2 to 0.3 c.c. of an 18-hour-old thioglycollate broth culture. Such inoculation (except for plates) is done with pipettes. Stabbing of the semisolid and the solid tube media is performed with a flamed wire.

2. All differential media used for classification data are to be incubated at 37° C. for at least 40 hours. None should be incubated at that temperature for more than 60 hours unless otherwise specified.

3. Only carbohydrates listed in the tables are inoculated in a small laboratory. A sugar-free thioglycollate broth (M-54) must be used as a control.

4. A 0.5 c.c. amount of a 0.04% brom cresol purple solution is pipetted into each carbohydrate tube, in order to determine the result of the fermentation after incubation. No indicator should be employed directly with the basic carbohydrate broths because they are often irreversibly reduced. The indicator must be added, therefore, after incubation.

5. Nagler plates (M-57), or any modification thereof, should be avoided in small laboratories. The lecithovitelline reaction is of diagnostic value only in the hands of those well experienced with it.

6. The ability of Clostridia to produce hydrogen sulphide is checked in three media simultaneously employed: iron-lactose-trypticase agar (M-56), iron-gelatin (M-52), and iron-litmus milk (M-51). The first and third of these media are incubated aerobically, the second anaerobically. Darkening or blackening of media other than these three is not considered an index of hydrogen sulphide production.

7. The reduction of nitrates to nitrites is determined in 48-hour cultures in thioglycollate nitrate broth (M-50).

8. Tryptone broth (M-1) is inoculated and incubated anaerobically for 48 hours for indol production.

9. Iron-litmus milk tubes (M-51) and all liquid media with thioglycollate are incubated aerobically.

10. The tables of differential reactions (Tables 156 to 159) contain 48-hour readings. This should explain some of the disparity between these and some other tabulations listing reactions recorded at time intervals other than 48 hours.

11. In vivo demonstration of bacteriemia and toxemia:

A chopped heart tube (M-12) is inoculated with approximately 0.5 c.c. of an 18-hour thioglycollate broth culture. This medium is then incubated aerobically according to the following outline:

Organism	*Time*	*Temperature*
Cl. tetani	3 to 5 days	37° C.
Cl. botulinum	5 to 7 days	30° C.
Cl. fallax	24 to 36 hours	37° C.
Remainder	48 hours	37° C.

(a) Traumatize the thigh muscles of a guinea pig by using slight pressure with a pair of large forceps or a hemostat.

(b) Denude the area of approximately 1 sq. cm. of hair.

(c) Cleanse with tincture of iodine, followed by absolute alcohol or acetone.

(d) Using a 20 gauge needle and a 5 c.c. syringe, inject 0.5 to 0.8 c.c. of the culture fluid into the thigh muscles.

(e) For *Cl. botulinum*, an intraperitoneal injection may be made using 0.5 c.c. of culture fluid. Forced feeding of the animal can be tried directly with cell-free filtrate, but is most dangerous.

(f) Hold all animals at least 7 days before declaring the result negative.

Table 156.—Reactions of Clostridia in 48 Hours

A. *Stormy Fermentation in Iron-Milk*

CLOSTRIDIUM	GLUCOSE	MALTOSE	SUCROSE	LACTOSE	SALICIN	MANNITOL	STARCH	GALACTOSE	GLYCEROL	HYDROGEN SULPHIDE	GELATIN LIQUEFACTION	NITRATE REDUCTION	INDOL PRODUCTION	ACROLEIN PRODUCTION	LECITHINASE	HEMOLYSIN	GUINEA PIG, LESION OR DEATH		SPORES
perfringens	+	+	+	+	V	–	V	+	V	V	V	V	–	+	+	+	+		O, sl S, C to St.
multifermentans	+	+	+	+	+	–	+	+	+	–	–	+	–	–	–	+	–		O, S, Ex.
butyricum	+	+	+	+	+	+	+	+	–	V	+	V	–	–	–	–	–		O, S, St.
pasteurianum	+	+	+	–	+	–	–	+	V	–	–	–	–	–	–	–	–		O, S, St.
beijerinckii	+	+	+	+	+	+	–	+	–	V	–	–	–	–	–	–	–		O, S, Ex.
aerofoetidum	+	+	–	+	+	–	–	+	–	+	+	+	–	–	–	–	V		O, St.

Key:
G: gas produced
+: positive
–: negative
V: variable
O: oval
R: spherical

S: swollen
St: subterminal
C: centric
Ex: excentric
T: terminal

(Blanks indicate sufficient strains have not been studied, or reports vary too greatly.)

TABLE 157.—REACTIONS OF CLOSTRIDIA IN 48 HOURS

B. *Acid, With or Without Clotting, With or Without Gas*

CLOSTRIDIUM	GLUCOSE	MALTOSE	SUCROSE	LACTOSE	SALICIN	MANNITOL	STARCH	GALACTOSE	GLYCEROL	HYDROGEN SULPHIDE	GELATIN LIQUEFACTION	NITRATE REDUCTION	INDOL PRODUCTION	ACROLEIN PRODUCTION	LECITHINASE	HEMOLYSIN	GUINEA PIG, LESION OR DEATH	SPORES
tertium	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	V	–	E, S, T.
fallax	+	+	+	+	+	–	+	+	–	+	–	+	–	–		–	+	O, S, St.
paraputrificum	+	+	+	+	+	–	+	+	–	V	G	–	–	–		–	–	R, S, T.
carnis	+	+	+	+	+	–	–	+	–	–	–	–	–	–		V	+	O, St – T.
feseri (chauvei)	+	+	+	+	–	–		+	–	+	+	+	–	–		+	+	O, S, St.
septicum	+	+	–	+	+	–		+	–	+	+	+	–	–		+	+	O, S, St.
sphenoides	+	+	–	+	+	V	V	+	–	+	–	V	V	–		V	–	R, S, T.
viscifaciens	+	+	+	+	+	+	+	+	+	V	–	+	–	–		–	–	
filiforme	+	–	+	+	–	–	–	–	–	–	+	–	–	–		–	–	R.

For *Key*, see Table 156.

TABLE 158.—REACTIONS OF CLOSTRIDIA IN 48 HOURS

C. *Acid-Digestion in Iron-Milk*

CLOSTRIDIUM	GLUCOSE	MALTOSE	SUCROSE	LACTOSE	SALICIN	MANNITOL	STARCH	GALACTOSE	GLYCEROL	HYDROGEN SULPHIDE	GELATIN LIQUEFACTION	NITRATE REDUCTION	INDOL PRODUCTION	ACROLEIN PRODUCTION	LECITHINASE	HEMOLYSIN	GUINEA PIG, LESION OR DEATH	SPORES
sporogenes	+	+	–	–	V	–	V	V	V	+	+	–	–	–	+	+	–	O, S, St.
novyi	+	+	–	–	–	–	–	V	V	+	+	–	–	–	+	+	+	O, S, St.
bifermentans	+	+	–	–	V	–	–	V	V	+	+	–	+	–	+	+	–	O, Sl, S, St.
sordelli	+	+	–	–	V	–	–	V	V	+	+	–	+	–	+	+	+	O, Sl, S, St.
lentoputrescens	V	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	–	R, S, T.
histolyticum	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	+	O, S, St.
parabotulinum	+	+	–	–	+	–	–	–	+	+	+	–	–	–	+	–	+	

For **Key**, see Table 156.

TABLE 159.—REACTIONS OF CLOSTRIDIA IN 48 HOURS

D. *No Change in Iron-Milk*

(Occasional Reduction of Indicator)

CLOSTRIDIUM	GLUCOSE	MALTOSE	SUCROSE	LACTOSE	SALICIN	MANNITOL	STARCH	GALACTOSE	GLYCEROL	HYDROGEN SULPHIDE	GELATIN LIQUEFACTION	NITRATE REDUCTION	INDOL PRODUCTION	ACROLEIN PRODUCTION	LECITHINASE	HEMOLYSIN	GUINEA PIG, LESION OR DEATH	SPORES
tetani	–	–	–	–	–	–	–	–	–	+	V	–	V	–	–	+	+	R, S, T.
tetanomorphum	+	+	–	–	V	–	–	V	–	V	V	–	V	–	–	+	–	R, S, T.
cochlearium	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	O, S, T.
capitovale	+	–	–	–	–	–	–	+	–	V	V	–	+	–	–	–	–	O, S, T.
difficile	+	–	–	–	+	V	–	–	–	–	–	–	–	–	–	+	+	O, S, St.

For *Key*, see Table 156.

All data obtained from differential reactions should be systematically entered on the work sheet. Comparison is then made with the tables of reference.

Occasional deviations from the tables will be encountered; in such cases, refer to the outstanding characteristics of the species in question, since individual, single enzymatic reactions may be at variance.

REFERENCES

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Humphreys, F. B.: J. Infect. Dis. **35**: 282, 1924.
McClung, L. S., and Toabe, R.: J. Bact. **53**: 139, 1947.
406th MGL Annual Reports, Prof. Sect., 1950, 1951, 1952, 1953, 1954.

Malleomyces Mallei
(Glanders Bacillus)

Morphology: Small, slender rods, 0.25 to 0.4 by 1.5 to 3.0 microns, occurring singly, but they may grow into long threads. Nonmotile.

Stain: Gram-negative, irregular staining, often bipolar.

Colonies: On Loeffler's blood serum, they are moist, opaque, slimy, with yellowish brown tinge. On glycerin veal agar, they are whitish, transparent. They acidify and coagulate milk. Cause glanders.

The medium of choice is glycerin potato veal agar formula M-38.

Slimy, tenacious colonies are characteristic. They develop in about one week. The principal characteristics of the organism are:

(a) No fermentation of carbohydrates, except occasional slight action on dextrose.
(b) Very unevenly staining, granular, gram-negative organisms. Use methylene blue stains.

SEROLOGY

Agglutination Method of Povitsky

(1) **Preparation of the Culture.**—Use a forty-eight-hour culture of *Malleomyces mallei* (the causative organism of glanders) on glycerin-potato-veal agar formula M-38.

Wash off the growth with a small amount of sterile physiologic saline. This gives a milky suspension.

Pour off the suspension.

Heat for one hour at 70° C.

This suspension may be kept in the ice box for two or more months.

To use, add enough sterile physiologic saline to make it only faintly cloudy.

This suspension should be tested against a known positive and a known negative serum before use.

(2) **Agglutination test** using patient's blood serum.—Dilute the serum to be tested 1 to 40 with sterile physiologic salt solution; that is, 1 part of serum to 39 parts of saline.

Place 3 c.c. test fluid (described above) in each of 5 test tubes.

Place in the first tube 0.1 c.c. of the 1:40 serum, making the dilution of serum in this tube 1:1,200.

Place in the second tube 0.075 c.c. serum diluted 1:40, making the dilution of serum in this tube 1:1,600.

Place in the third tube 0.06 c.c. serum diluted 1:40, making the dilution of serum in this tube 1:2,000.

Use positive serum in the fourth tube, and negative serum in the fifth tube.

Shake.

Place the tubes in a water-bath at 37-40° C. for two hours.

Very active reactions may be read at the end of one hour.

Place in the ice box overnight.

If a reaction occurs, the upper part of the liquid is clear, while a veil-like sediment is found. A negative reaction shows the sediment in a definite "button" at the bottom of the tube, the fluid above being cloudy.

Glanders occurs in man as an acute or chronic systemic infection and as a cutaneous disorder. Most of the cases reported in the past have been due to direct or indirect contact with infected horses. Accidental infections have been recorded among laboratory workers. Agglutinin titers higher than 1:320 are considered diagnostic. The complement fixation test is a less sensitive index, but is more specific. When positive in dilutions of 1:20 or higher, it is diagnostic of infection with *M. mallei.*

Mallein Test

Another diagnostic aid found quite useful is a modification of the mallein skin test, permitting its safe use in human beings. One-tenth (0.1) c.c. of commercial mallein in a dilution of 1:10,000 is injected intradermally into the skin of the forearm. The test is read at 24 and 48 hours. Specific positive tests reach maximum erythema, 10 to 20 mm. in diameter, after about 18 to 24 hours and fade only slightly if at all by the end of 48 hours.

ANIMAL INOCULATION

Straus Test

Inoculate several male guinea pigs with increasing amounts of the culture. A swollen, red, and tender scrotum on the second or third day is a sign of a positive reaction.

MELIOIDOSIS

The synonym of this disease is Stanton's disease which is caused by *Malleomyces pseudomallei.* It occurs in Burma, the Malay States, and Ceylon. It bears many resemblances to tularemia because both are diseases of rodents which accidentally attack man. It closely resembles glanders. It was reported by Stanton and Henessy in 1917, although earlier Whitmore, in 1912, found the bacterium in autopsies of babies in Rangoon. Krishnaswami found 200 cases in Rangoon. Other cases have been reported in Singapore.

The first case reported in the Western Hemisphere is that by McDowell and Varney,[1] showing ulcers and sinuses of the buttock and thigh of eight years' duration.

The *Bacillus whitmori (Malleomyces pseudomallei)* closely resembles *M. mallei.* The bacillus is small and occurs in very large numbers in all acute lesions of the disease. With Leishman's stain, bipolar staining is commonly seen. It resembles the glanders bacillus in colonies but is more actively motile.

Bacteria from rough colonies average 2 by 0.6 microns, from smooth colonies, 5 to 6 microns. They show parallelism and are usually arranged in an irregular network. Growth is strictly aerobic. The organisms grow rapidly and profusely on all commonly used media at 24° to 37° C. Colonies of the smooth form are entire, convex, and mucoid, and often grow to a diameter of 8 mm., are initially grayish white, but change to a deep brown. They are surrounded by a narrow inner zone of complete hemolysis and a larger zone of diffuse hemolysis on blood agar plates (M-28 and M-29).

Differential Diagnosis

Miller and associates[2] recommend the following procedure for the isolation of *Malleomyces mallei* and *pseudomallei:*

(a) Cultivation on eosin-methylene blue agar (M-71 or M-72) with 4% glycerol.

(b) If more heavily contaminated, collect specimen, before plating, in saline with 1,000 units of penicillin per c.c., and incubate for 3 hours at 37° C.

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[1] McDowell, Frank, and Varney, Philip L.: J. A. M. A. 134: 361-362, May 24, 1947.

[2] J. Bact. 55: 115, 1948.

(c) Agglutination which, however, does not differentiate the two species.
(d) Animal inoculation, especially of hamsters.
(e) If feasible, oxidase reaction (R-18).

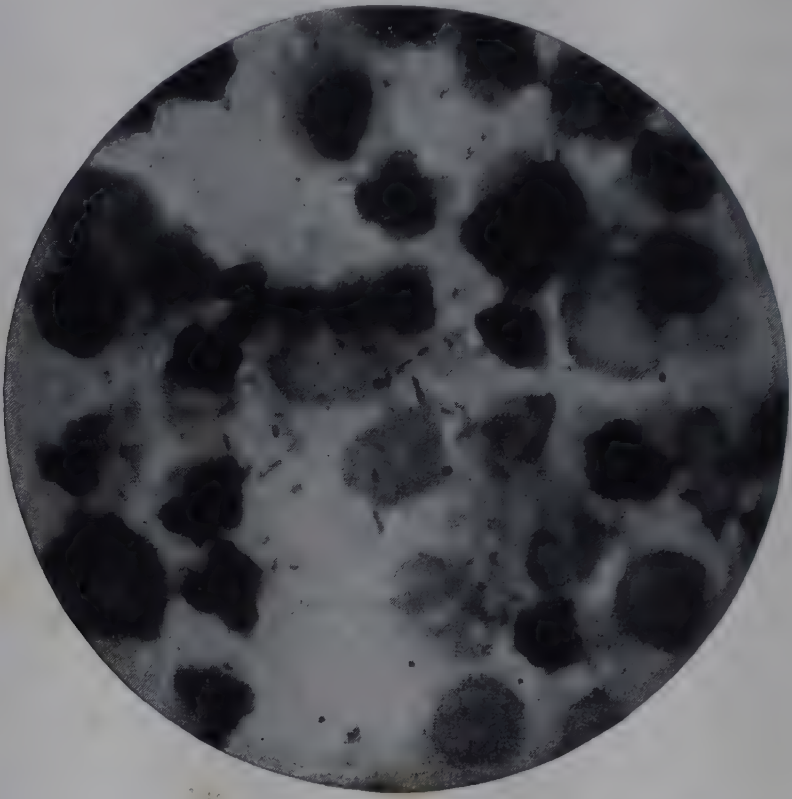

Fig. 374.—Photomicrograph showing direct smear from cerebrospinal fluid of case of influenza meningitis.

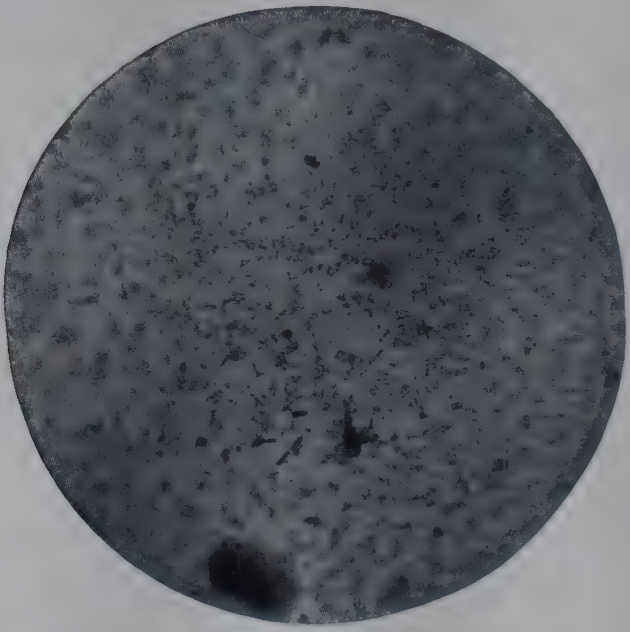

Fig. 375.—Photomicrograph of pure culture of influenza bacilli. (From Levinson: Cerebrospinal Fluid in Health and in Disease, ed. 3, St. Louis, 1929. The C. V. Mosby Co.)

HEMOPHILUS

The hemophilus group is a rather heterogeneous mixture of gram-negative microorganisms. In human diseases, *H. influenzae* and *H. pertussis* are important, while *H. haemoglobinophilus* and *H. hemolyticus* may cause diagnostic difficulties.

H. influenzae requires chocolate agar M-31, but grows well also on blood agar with human or rabbit blood M-28, M-29. It is differentiated from *H. hemolyticus* in that the latter grows on media containing yeast but no hemoglobin.

H. pertussis requires glycerin potato blood agar M-39, for its growth. It forms hemolytic, pearly, small colonies in 48 hours. It is easily differentiated from *H. para-pertussis* and *Br. bronchiseptica* by its inability to grow on agar without blood.

Hemophilus Influenzae

Morphology: Rods, very small 0.2 to 0.3 by 0.5 to 2.0 microns, occurring singly and in pairs, occasionally in short chains, and at times in long thread formation. Frequently show a marked tendency to bipolar staining. Nonmotile.

Stain: Gram-negative.

Colonies: On blood agar, they are small, circular, transparent, homogeneous, entire. Cause meningitis, pleurisy, and other inflammations.

Hemophilus Pertussis

(Bacillus of Bordet and Gengou)

Morphology: Short, oval rods, 0.2 to 0.3 by 0.5 to 2.0 microns, occurring singly, sometimes in pairs, frequently showing tendency to bipolar staining. Nonmotile.

Stain: Gram-negative.

Colonies: On blood agar, they are small, transparent, entire. Causes whooping cough.

Agglutinating antisera can be prepared against *H. pertussis* by injecting rabbits twice a week with 0.5 c.c., 0.7 c.c., 1.0 c.c., 1.0 c.c., 2.0 c.c., and 2.0 c.c. quantities (in that order) of a suspension of the density of tube 4 of the nephelometer (page 1477). The suspension is prepared by washing 48-hour-old cultures on glycerin potato blood agar formula M-39 with 1:10,000 Merthiolate in saline.

Mix 0.1 c.c. doses of serum dilutions with 0.1 c.c. amounts of the antigen, shake for 3 minutes, then add 0.5 c.c. saline to each tube and read the results. Look for agglutination or lack of agglutination. Only 3 plus agglutination is considered positive.

Agglutination and complement fixation tests are carried out with the serum of the patient. They are not widely used.

OPSONOCYTOPHAGIC INDEX

Kendrick and associates[1] described this test, using 20 billion *H. pertussis* preserved in 1:10,000 Merthiolate containing saline.

The performance and reading of the test are the same as in the Huddleson opsonocytophagic test for the diagnosis of brucellosis, pages 1546 and 1573.

Hemophilus Conjunctivitidis

(Koch-Weeks Bacillus)

Morphology: Rods, small, 0.2 to 0.3 by 0.5 to 2.0 microns, with rounded ends, occurring singly. Nonmotile.

Stain: Gram-negative.

Colonies: On blood agar, they are small, transparent, entire. Cause infectious, contagious conjunctivitis.

According to *Bergey's Manual of Determinative Bacteriology* (1948), this is the same organism as *H. influenzae.*

Hemophilus Duplex

(Diplobacillus of Morax and Axenfeld, Moraxella Lacunata)

Morphology: Short rods, 0.4 to 0.5 by 2.0 microns, occurring singly and in pairs and short chains. Ends rounded or square in the chains. Nonmotile.

Stain: Gram-negative.

Colonies: On blood agar, they are small, circular, transparent, entire. Cause catarrhal conjunctivitis.

Hemophilus Ducreyi

(Bacillus of Soft Chancre)

Morphology: Small rods, 0.4 by 1.5 microns, with rounded ends, occurring singly and in short chains. Nonmotile.

---

[1] J. Infect. Dis. 60: 302, 1937.

Stain: Gram-negative.

Colonies: On blood agar, they are small, grayish, glistening, showing a slight zone of hemolysis around the colony in three or four days. Best growth on clotted rabbit, sheep, for human blood, heated to 55° C. for fifteen minutes. Cause "soft chancre."

TABLE 160.—DIFFERENTIAL DIAGNOSIS OF HEMOPHILUS*

	BLOOD AGAR PLATE (X)	SATELLITE PHENOMENON (V)	INFUSION AGAR
H. pertussis (dextrose −; indol −)	+	−	+
H. parainfluenzae	+	+	+
H. hemolyticus			
H. ducreyi	+	−	−
H. canis (haemoglobinophilus)			
H. influenzae (usually dextrose +; indol +)	+	+	−
H. Koch-Weeks (indol −; agglutinates human red blood cells)			
H. suis (maltose +; sucrose +)			

*Strains cultivated in the laboratory for at least 3 subcultures.

Summary of Methods of Laboratory Diagnosis of Chancroid

The methods commonly used in the laboratory diagnosis of chancroid are biopsy, cultures, and skin tests. Small lesions do not permit biopsy. Culture results are useful in the majority of cases. Smear methods are also useful. Skin tests are dubious. Autoinoculation has been suggested but it is, in our opinion, too dangerous to be used. Emphasis, too, in this diagnosis must be placed upon the results of staining of smears by Pappenheim and Sellers' method. The Ducrey bacilli show a tendency to bipolar staining which gives some of them the "closed safety-pin" appearance. Greenblatt and others have stressed the "school of fish" arrangement in which the bacilli are grouped in long parallel columns between cells or shreds of mucus.

The Skin Test as a Method of Diagnosis of Chancroid

The Ito-Reenstierna[2] Reaction

It has been shown that individuals having chancroid infection are sensitive to the intradermal injection of killed cultures of *H. ducreyi.* This phenomenon is known as the Ito-Reenstierna reaction. Sterilized, diluted bubo pus from chancroid patients has been used by Cole and Levin,[3] and others, but in comparative studies by Greenblatt and Sanderson,[4] it has been shown that this material is much less reliable than an antigen made from a killed suspension of *H. ducreyi.* Dulaney[5] has substantiated the findings as to the value of the test with a bacillary antigen in diagnosis.

The skin reaction does not become positive until 10 days to 2 weeks following the appearance of the initial lesion, and remains positive for many years and perhaps for life. These facts must be given consideration in the interpretation of the findings. In the differentiation of chancroid from other venereal ulcers and buboes, it is well to remember that the appropriate procedures to rule out granuloma inguinale, lymphogranuloma venereum, fusospirochetosis, gonorrhea, and carcinoma should be performed.

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[2] J. Infect. Dis. 60: 302, 1937.
[3] J. A. M. A. 105: 2040, 1935.
[4] Arch. Dermat. & Syph. 36: 486, 1937.
[5] Am. J. Syph. 21: 667, 1937.

Preparation of Antigen for Skin Tests.—Some strains of *H ducreyi* will grow on the surface of infusion blood agar slants (0.1% glucose, and 3 to 5% whole blood) in CO_2 atmosphere as advocated by Hunt.[6] When the transplant has been made, the side of the tube is gently heated in a Bunsen flame, and the tube immediately stoppered with a rubber stopper. After 48 hours' incubation at 37° C. the growth is removed from the slant with saline and centrifugated. The supernatant fluid is removed, and the sediment resuspended in saline and killed by heating at 60° C. for 40 minutes. Phenol 0.3% or Merthiolate 1:10,000 can be added as a preservative. The antigen should be vigorously shaken to break up the clumps.

Method of Sanderson and Greenblatt.—All the strains of *H. ducreyi* isolated by these authors grew poorly or not at all on the surface. To obtain a satisfactory antigen, the following method was employed: To each slant of beef infusion agar M-15 is added *onto the surface* 1 c.c. of human defibrinated blood, inoculum of culture is made into the blood, following which the tube is gently heated on the side and then stoppered with a rubber stopper to reduce the oxygen tension. After 3 days' incubation at 37° C., the blood is removed and laked in 25 c.c. of sterile distilled water and centrifugated. The sediment is rewashed until free of hemoglobin—usually twice is sufficient. The sediment from each culture tube is then added to 10 c.c. of physiologic saline, killed at 60° C. for 40 minutes and preserved with 0.3% phenol or Merthiolate 1:10,000.

A *positive* reaction to an intradermal injection of 0.1 c.c. of antigen is manifested by an area of induration surrounded by an erythematous halo at the site of injection. The induration should measure 7 mm. in diameter in positive cases. Greenblatt and Sanderson* have noticed that occasionally the induration may be minimal, but that in such cases the erythema must measure at least 14 mm. before the reaction may be considered as positive. Any smaller reaction is a doubtful test and in such cases the injection should be repeated after a few days. Strong reactions sometimes have a central pustular area. Generalized reactions have not been observed. Frank negatives show nothing but traumatic effect of the injection.

PASTEURELLA

Pasteurella Avicida

(Bacillus Cholerae Gallinarum)

Morphology: Small rods, 0.25 to 0.4 by 0.5 micron, with rounded ends, occurring singly. Nonmotile.

Stain: Gram-negative.

Colonies: Transparent bluish, becoming semiopaque. The slant is thin, white, glistening. They cause fowl cholera.

Pasteurella Suilla

(Pasteurella Suiseptica)

Morphology: Rods, 0.4 to 0.5 by 1.0 to 1.5 microns, occurring singly. They are nonmotile.

Stain: Gram-negative.

Colonies: Scant growth on agar, growth becoming slimy. They cause swine plague.

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*Greenblatt and Sanderson: J. M. A. Georgia 27: 218-219, 1938; ibid. 27: 320, 1938.

[6] Proc. Soc. Exper. Biol. & Med. 33: 293, 1935.

Pasteurella Bollingeri (Boviseptica)

Morphology: Rods, 0.5 by 0.6 to 1.0 micron, occurring singly. They are nonmotile.

Stain: Gram-negative.

Colonies: Grayish white, slightly raised, undulate. They cause hemorrhagic septicemia in domestic cattle, hogs, and horses.

Pasteurella Pestis
(Bacillus Pestis)

Morphology: Rods, 0.5 to 0.7 by 1.5 to 2.0 microns, occurring singly. Nonmotile.

Stain: Gram-negative.

Colonies: Grayish white, translucent, iridescent, undulant. Growth on agar is grayish, viscid, thin, moist, translucent.

They cause plague in man, rats, and ground squirrels. Transmitted from rat to rat and from rat to man by the infected rat flea.

Devignat[1] classifies plague bacilli as:

	GLYCERIN	NO_2 PRODUCTION
Oriental tyye	0	+
Antic type	+	+
Medieval type	+	0

Human material suspected of containing Pasteurella must be handled with extreme care. In fact, many State laws forbid the handling of such material in other than Public Health laboratories. The following procedure is recommended[2]:

> "To the Officers of the Public Health Service and State and Local Health Officers:
>
> "Owing to the appearance of plague in several American ports it is important that all cases of suspected plague, both in man and animals, be subjected to a bacteriologic examination.
>
> "1. The following material from persons or rodents suffering from plague may be sent to laboratories:
>
> **Human Cases (Living)**
>
> "(a) Pus or gland fluid from buboes aspirated by syringe or collected after incision, on agar slants.
> "(b) Portions of tissues affected, removed at operation, in sterilized bottles, securely stoppered.
> "(c) Blood specimens, in sterilized sealed glass ampules or test tubes.
> "(d) Cultures of suspected organisms, on agar slants.
>
> **Human Cases (Necropsy)**
>
> "(a) Portions of the affected tissues—preferably bubo, lung, and spleen—in sterilized glass bottles, securely stoppered.
>
> **Rodents**
>
> "(a) The whole rodent carcass, in fruit preserving jar.
>
> "2. Do not place tissues or rodents in a preservative. The bacteriologic diagnosis of plague rests upon the production of the disease in laboratory animals, and the isolation and growth of the causative organism, *Bacillus pestis*. Any preservative that kills this organism will defeat the purpose of the exami-

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[1] Bull. W. H. O. 4: 247, 1951.

[2] Pub. Health Rep. 35: No. 37.

nation. If decomposition of the specimen is feared, it may be placed in a tight container and this in turn surrounded by ice in a larger container, preferably of wood. Every specimen should be plainly marked, preferably by ordinary pencil, showing the date and the exact location from which it was taken.

"3. The shipper must make certain that the specimen is packed in such manner as to prevent possible danger to those handling the same, provided the package is properly handled.

"In this connection it is necessary that specimens be wrapped in sufficient cotton, or other absorbent material, to prevent leakage of fluid from the container should the glass be broken.

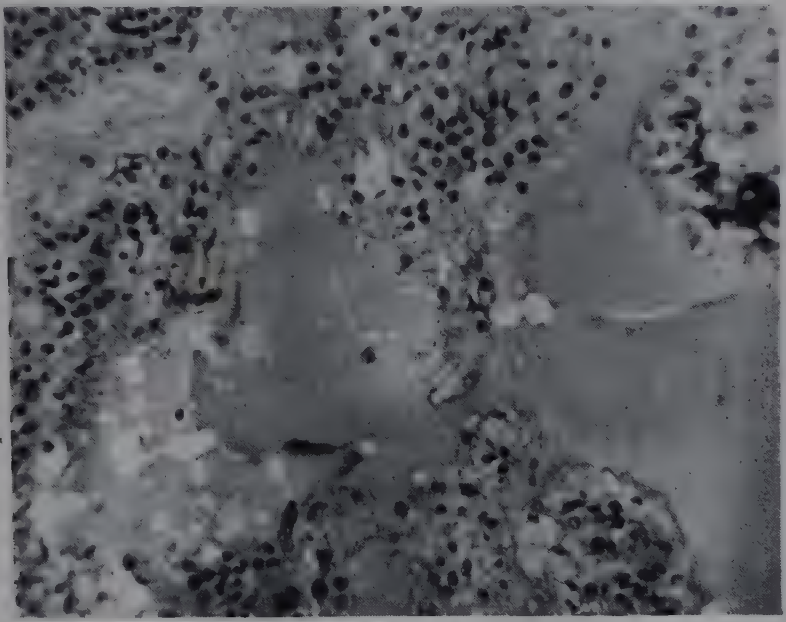

Fig. 376.—Plague bacilli in lung lesion (×300). (Courtesy Dr. Oscar Felsenfeld.)

"The following Instructions should be **explicitly observed.**

"1. **Ship by express**—Federal laws prohibit the shipping of plague-infected material, or cultures, by mail.

"2. Do not make packages too small, as small packages are more likely to be lost in transit, or overlooked.

"3. Each package should be marked as follows:

Notice
This package contains perishable specimens
for bacteriological examination
Please Expedite."

Laboratories carrying out tests for *Past. pestis* base their examination on the following properties of the plague bacillus:

(a) Bipolar, short, ovoid, gram-negative bacilli.

(b) Growth on agar (M-15) with 3 to 3.5% sodium chloride.

(c) Acid in dextrose, mannitol, salicin; no action on lactose, sucrose, dulcitol, or sorbitol.

(d) Indol negative.

(e) Does not reduce methylene blue.

(f) No motility at 22° C. in 18 hours.

(g) Animal inoculation tests.

The above reactions are carried out in media with fermentable substances M-5, tryptone water M-1, or cystine trypticase medium M-6.

SEROLOGY

There are capsular and somatic antigens.

Precipitin tests with animal tissue, similar to Ascoli's test for the diagnosis of anthrax (page 1547), are used.

The agglutination test is difficult to do because of the self-agglutinating qualities of most strains.

Specific phages are used to differentiate types of plague bacilli.

Plague phage, dried on paper strips, may also serve to identify plague bacilli (Cavanaugh and Guan*) when put on plates inoculated with the suspected strain.

Pasteurella Tularensis

Morphology: Small rods 0.2 by 0.3 to 0.7 micron, occurring singly. They are nonmotile.

Stain: Gram-negative.

Colonies: They grow on media containing egg yolk, blood, blood serum and dextrose. Sterile rabbit spleen, fresh, aids the growth.

They are agglutinated by immune tularemia serum. Cause tularemia or "rabbit" fever.

Past. tularensis does not grow on ordinary media. Cystine agar M-30 and, to a certain extent, egg media may be used for its cultivation.

Tularemia is an acute, often fatal disease of rodents caused by *Pasteurella tularensis.* It is highly infectious and is transmitted to man from rodents by the bite of a flea or tick, or by contamination of the skin or conjunctiva with tissues or body fluids of infected rodents, flies or ticks. The site of infection in man is usually marked by a necrotic, punched-out ulcer, which is associated with a regional lymphadenitis that tends to chronicity. The disease begins with a sudden chill, followed by an irregular fever, lasting two or three weeks. Convalescence is prolonged.

McCoy and Chapin in 1912 found the causative organism in squirrels, and named it *Bacterium tularense,* cultured it, transmitted the infection to various rodents by feeding, nasal inoculation, and injection of blood, and reported positive complement fixation and agglutination for human sera. Francis identified, in 1919, the deer-fly fever of Utah with the plaguelike disease of rodents of California, and in 1921, gave the name of "tularaemia" to the disease on account of the bacteriemia. He isolated *Past. tularensis* from fly-bitten human cases, wild jack rabbits, and one ground squirrel, and demonstrated agglutinins in human cases, and transmitted infections in laboratory animals with the deer fly, *C. discalis.* Since that time many cases have been described in the United States.

The occurrence of this disease in man depends on the presence of the disease in wild rodents, especially rabbits, and is often known to be associated with the discovery of sick and dead rabbits in the vicinity. Scratches, abrasions, thorn punctures, etc., have been reported at the site of infection. Infection is acquired from infected tissues or body fluids, and may occur in connection with dressing or handling rabbits, woodchucks, or laboratory animals or with the handling of ticks or flies. Infection from man to man has not been reported either by contact, operative procedures, or insects. The disease is found especially in rural districts, among those exposed to ticks and flies and especially those handling wild rabbits, such as hunters, marketmen, housewives, and cooks. Laboratory infections are frequent.

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*Am J. Clin. Path. 23: 619, 1953.

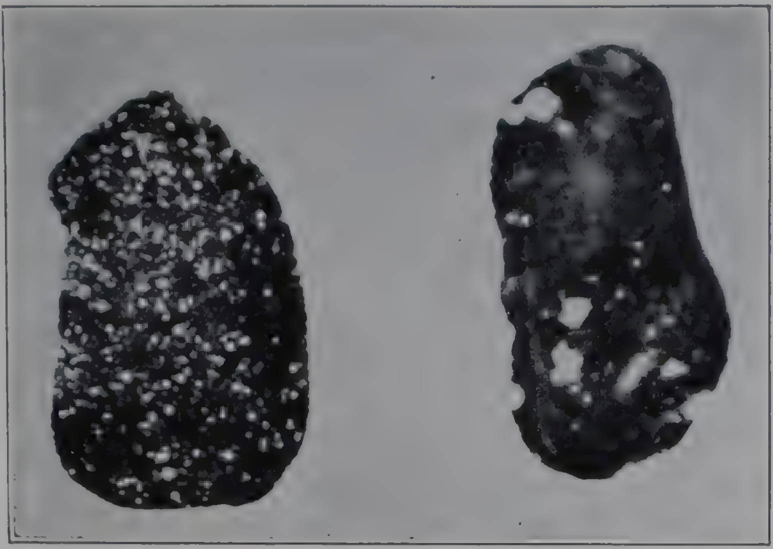

Fig. 377.—Guinea pig spleens, showing acute (left) and subacute (right) lesions of tularemia. (Francis.) (Army Medical Museum No. 40325.) (From Simpson, W. M.: Tularemia, Paul B. Hoeber, 1929.)

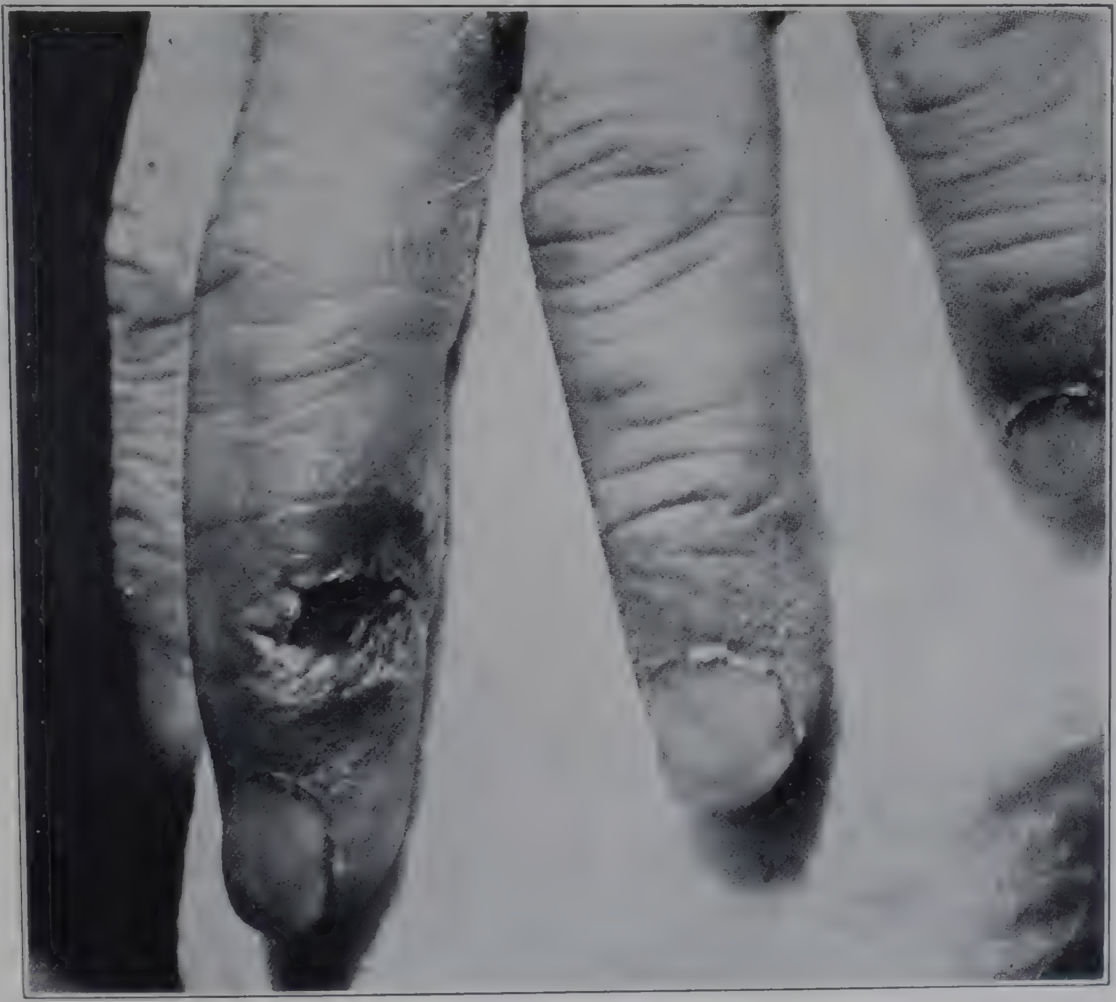

Fig. 378.—Ulcer of finger nineteen days after onset in a market man who dressed rabbits. (Brown and Hunter.) (Stitt: Diagnosis and Treatment of Tropical Diseases, P. Blakiston's Son and Co.)

Immunity is lasting in man and may be connected with the long persistence of agglutinins. There is no record of a second attack. Francis described five types of this disease: *ulceroglandular tularemia, oculoglandular tularemia, glandular tularemia, typhoidal tularemia,* and *ingestion tularemia.*

Diagnosis.—In connection with the agglutination test, which will be described below, it must be remembered that cross-agglutination between the sera of tularemia and undulant fever and their causative organisms is important. There is a marked difference in titer for *Past. tularensis* and the *melitensis-abortus* group. Blood of tularemia patients has the higher titer. Practically equivalent titers necessitate agglutinin-absorption tests.

The rabbit is the animal of choice for the development of an antiserum. Antigen is a 72-hour culture washed off with physiologic saline containing 0.2 per cent commercial Formalin, then centrifuged and the sediment suspended in physiologic saline containing 0.2 per cent commercial Formalin.

The *Pasteurella tularensis* cannot be successfully demonstrated in stained smears or sections of pathologic tissue of man, so that this method of identification is not practicable. The usual method of isolation of the organism is by animal inoculation, as described below.

In connection with the diagnosis, attention is called to the Schilling dif-ferential blood counts of three cases reported by the author.[1] (See pages 798, 799.)

Intradermal Test

A successful intradermal reaction was reported by Foshay.[2] This reaction has the appearance of a positive tuberculin reaction. It shows up in about 48 hours. Foshay test solution is made from virulent strains of *Past. tularensis.* This suspension is oxidized for about five hours with nitrous acid and then adjusted to a turbidity equivalent to 100 p.p.m. of fuller's earth. The standard test dosage of this suspension is 0.05 c.c. The proper time to read the response is at the forty-eighth hour after administration. Hypersensitivity of the skin is a notable feature of tularemia; therefore, caution must be used in selecting an antigen which affects the toxicity of heat-killed organisms for skin testing.

Bacteriologic Diagnosis by Animal Inoculation

This is accomplished by injecting a guinea pig intraperitoneally with blood obtained by venous puncture. This blood is defibrinated and mixed with an equal volume of physiologic saline solution. Inject about 5 c.c. of this defibrinated diluted blood. If *Pasteurella tularensis* is present, the guinea pig dies between the fourth and sixth day and the autopsy will show lymph nodes in the groin, enlarged, soft, and caseous. Spleen and liver are studded with multiple areas of white necrosis. Splenic and hepatic lesions sometimes so minute as to require hand lens for identification will be present. If no lesions are detected in animals which die in the usual time, transfers should be made to fresh animals.

In the case of other material, such as pus from the site of the fly bite or tick bite, or from the patient's suppurating glands, or tissue from a wild rabbit's spotted spleen or liver, this should be injected subcutaneously on the abdomen of guinea pigs or rabbits. These animals will die within a week if *Pasteurella tularensis* is present. They will show the same lesions as noted in the case of injection of blood into guinea pigs.

It is noteworthy that material from the dead animal's lymph nodes, spleen, or liver, rubbed upon the shaved abraded skin of another guinea pig or rabbit, will cause death within a week with the same typical lesions in the lymph nodes, spleen, and liver, and thus the infection may be propagated for an indefinite number of passages through guinea pigs or rabbits.

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[1] Gradwohl, R. B. H.: J. Missouri M. A., pp. 123, 124, March, 1933.

[2] Foshay, Lee: J. Infect. Dis. 51: 286: 1932.

Agglutination Test

Agglutination tests are highly successful in the diagnosis of tularemia. These tests will be worthless in the first week, but constantly positive in the second week. The test remains positive for seven weeks, the titer gradually increasing from the fourth week to the seventh, with a maximum of 1:1280 or 1:2560. It begins to fall in titer in the eighth week; but agglutinins persist for many months after recovery has occurred. Francis states that the agglutinins fail to disappear entirely in any case, even for ten to thirty years after recovery. At the end of the first year, the average titer is about 1:160. The final titer-level to which all cases seem to fall, even after ten, fifteen, or twenty years is 1:160, 1:80, or 1:40. Cross agglutination of *Brucella abortus* and *Brucella melitensis* may be found in many human tularemia sera. Also note that human brucellosis sera may show cross agglutination of *Pasteurella tularensis.* Therefore, if tularemia is suspected, agglutination should be made not only for *Pasturella tularensis* but also for *abortus* and *melitensis.* The titer with *Pasteurella tularensis* will be higher than with *abortus* and *melitensis* antigen in a case of tularemia.

One attack of tularemia confers permanent immunity, the agglutinins remaining in the blood serum. The titer acquired by the original attack does not become elevated by exposure to the disease.

If the blood serum of the case is diluted according to the figures given above for the length of time of infection, an agglutination test may be performed in the usual manner. (See pages 1528 ff.)

The antigen, or killed culture of *Pasteurella tularensis,* is made as described above. The patient's serum is diluted with physiologic saline. The test is performed by adding 0.5 c.c. of the patient's diluted serum to 0.5 c.c. of the antigen. Mix thoroughly and place in the incubator for eight to twelve hours. A positive reaction is indicated by the formation of a sediment consisting of agglutinated bacteria. The supernatant liquid is clear. A control of a mixture of saline and the antigen, and one containing diluted serum only, should also be made. It should show no sediment. Negative results are cloudy with no sediment.

Opsonocytophagic Test for Tularemia and Brucellosis
(Method of George A. Hunt)

Hunt[1] uses the following procedure in the diagnosis of tularemia. This procedure is similar to the opsonocytophagic test described by Gould and Huddleson[2] and Evans.[3] (See page 1545.)

Five c.c. of blood are added to 0.2 c.c. of 20% sodium citrate in a sterile tube. A mixture of equal parts (usually 0.1 c.c.) of this citrated blood and a saline suspension of *Br. abortus* or *Past. tularensis* organisms (living 48-hour culture or Formalin killed and washed free from Formalin just before use) equivalent in turbidity to 500 parts per million of kaolin in water, is incubated 30 minutes at 37° C. and the sedimented cells spread on a slide and stained according to Huddleson's or Evans' technic or any good blood stain. (See page 1545 for a second method.)

Hunt states that on several occasions he has been unable to obtain fresh citrated blood specimens before the polymorphonuclear leukocytes had disintegrated. In these instances it is possible to obtain results comparable to the opsonocytophagic test if one employs one volume (0.1 c.c.) of fresh citrated blood from a normal individual (5 c.c. blood added to 0.2 c.c. 20% citrate), one volume of plasma or serum from the patient, and one volume of the saline suspension of *Br. abortus* or *Past. tularensis* equivalent to 500 p.p.m. kaolin in turbidity. Controls are made using equal volumes of the normal citrated blood and the bacterial suspension to be certain that no phagocytosis takes place in the normal blood.

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[1] Hunt, George A., formerly Bacteriologist of the Laboratory Department of the St. Louis City Hospital: Personal communication.

[2] J. A. M. A. **109**: 1971, 1937.

[3] Pub. Health Rep. **52**: 1419, 1937.

Parallel tests were made as follows: (1) fresh citrated blood from the patient plus bacterial suspension, (2) plasma of patient plus normal citrated blood plus bacterial suspension, and (3) serum of patient plus normal citrated blood plus bacterial suspension. Results of tests made on sera and plasma of 25 patients all gave results comparable to the tests made using the technic described by Huddleson.

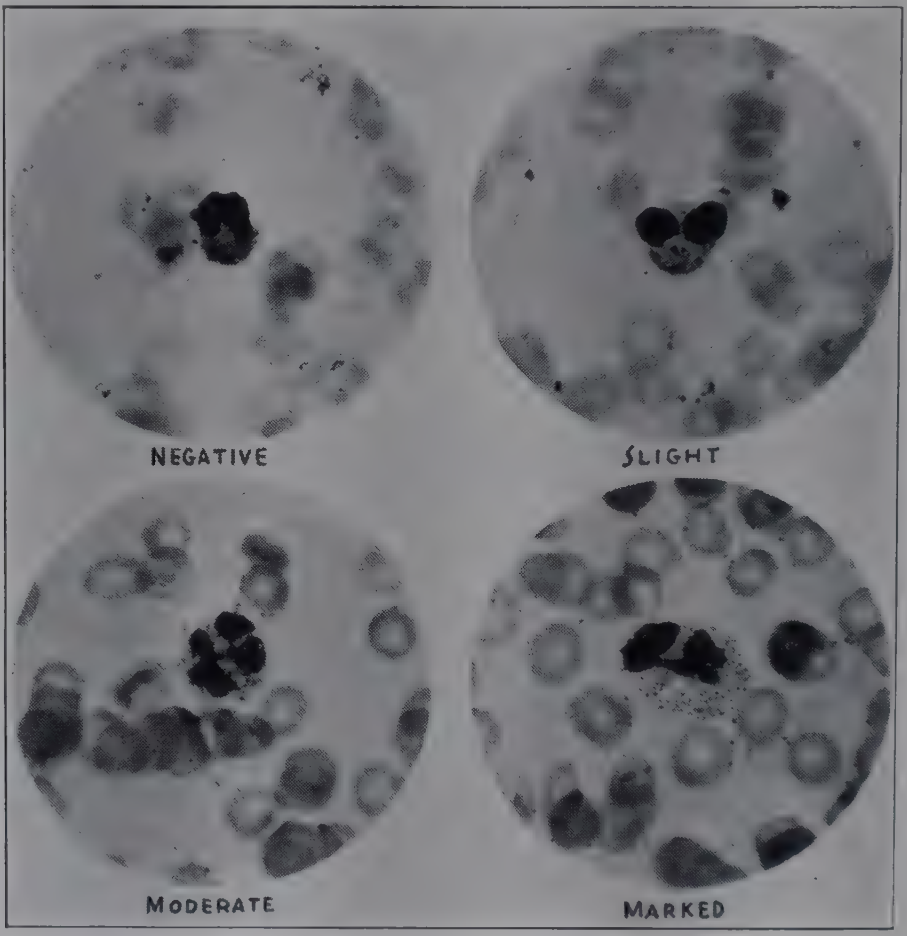

Fig. 379.—Positive and negative opsonocytophagic tests for tularemia and brucellosis.

Hunt has used this modified procedure only when it was impossible to obtain fresh citrated blood from a patient. It is difficult, however, for some laboratories to obtain fresh samples when most of their specimens are received by mail. The modified procedure may prove of value to central state laboratories.

For normal blood he has employed volunteers of the O-Group (Group IV Moss). He has not encountered hemagglutination and hemolysis and does not know whether this would interfere with the test. A few tests using fresh citrated rabbit blood in place of fresh citrated human blood gave satisfactory results.

Attention is called to the test because of the fact that many seem to have forgotten the work of Wright and the principle of opsonization and consequently hesitate to utilize it.

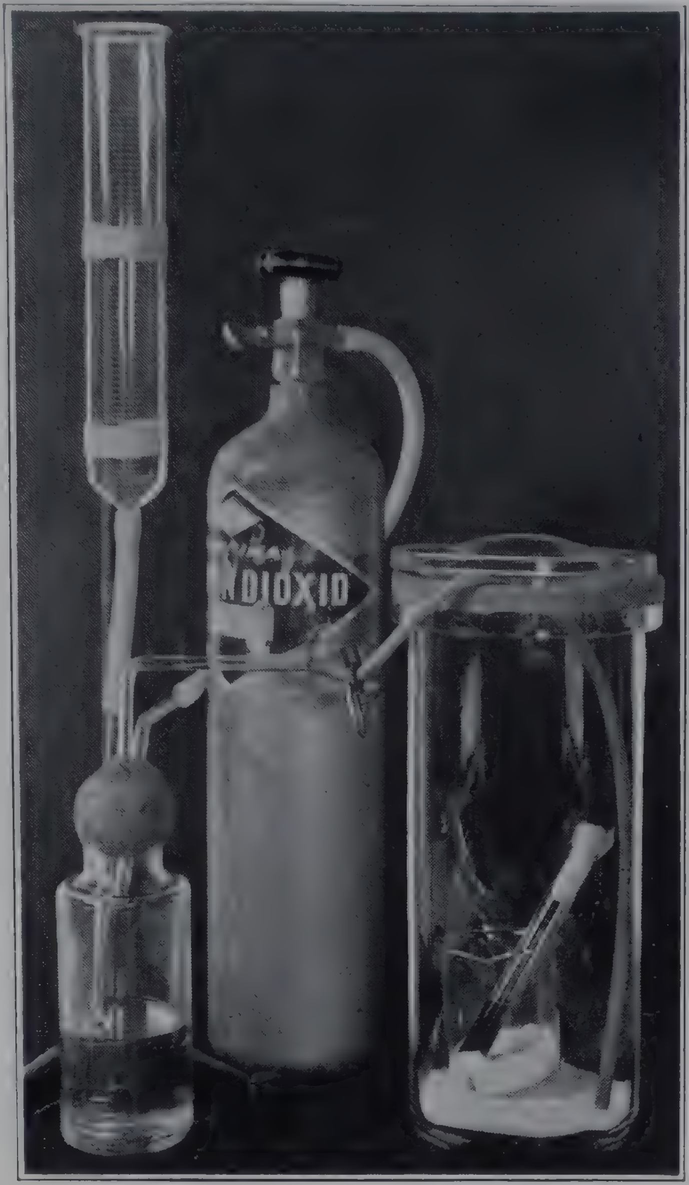

Fig. 380.—Apparatus used to produce a 10 per cent carbon dioxide atmosphere for culture of Brucella. (From Gould and Huddleson: J. A. M. A., Dec. 11, 1937.)

Pseudomonas Aeruginosa

(Bacillus Pyocyaneus)

Morphology: Rods, 0.5 by 2.0 microns, occurring singly, in pairs, short chains, and occasionally in thread formation. Motile by means of one to three polar flagella.

Stain: Gram-negative.

Colonies: Large, spreading, grayish with dark center and translucent edge, irregular. The medium becomes a greenish color, later turning brown. The blue color can be shaken out with chloroform. Dextrose is the only common sugar fermented, with acid production only. They may cause green pus formation.

The medium of choice for *Ps. aeruginosa* is the blood agar plate. Transfer colonies to agar slants M-13 for observation of pigment production.

Serratia Marcescens

(Bacillus Prodigiosus)

Morphology: Rods, 0.5 by 0.5 to 1.0 micron, occurring singly and occasionally in chains of 5 or 6 elements. Motile, with two to four peritrichous flagella.

Stain: Gram-negative.

Colonies: Circular, thin, granular, gray becoming red, first in the center, and later spreading to the periphery. The entire growth takes on a fuchsin-red color with a metallic luster. Nonpathogenic. They form a small amount of CO_2 gas in dextrose.

VIBRIO

Vibrio Comma

(Vibrio Cholerae)

Morphology: Slightly curved rods, 0.4 by 2.0 microns, occurring singly and in spiral chains. Motile, possessing one to two polar flagella.

Stain: Gram-negative.

Colonies: Circular, whitish, brown, moist, glistening, translucent, slightly raised, entire. Turn litmus milk alkaline at the top and slightly acid at the bottom; do not coagulate the milk, but peptonize it. They form acid in dextrose. They are found in the intestinal contents of cholera patients and carriers.

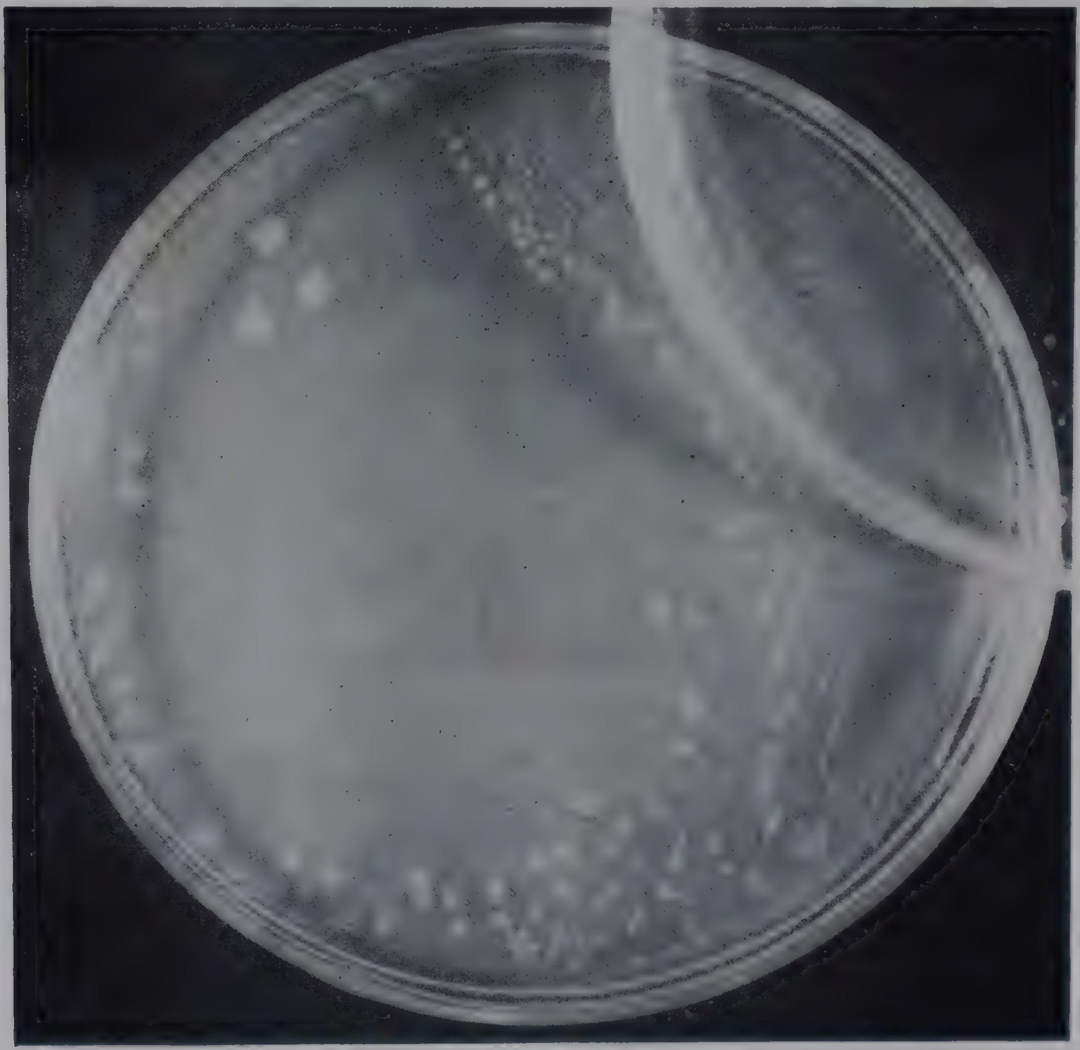

Fig. 381.—*Vibrio comma* colonies on D.E.C. plate of Panja and Ghosh. (Courtesy Dr. Oscar Felsenfeld.)

Cholera vibrios grow well on alkaline media. The true *V. comma* has the following properties:

(a) Fermentation of mannose and sucrose, but not arabinose, with the production of acid, but no gas.

(b) Indol and cholera red positive (R-12) and R-16).

(c) Voges-Proskauer negative (R-14).

(d) No beta-hemolysis of goat cells (R-3).
(e) Agglutination with specific sera.

Media for the preservation, isolation, and identification are given in the section on "stool examination." The same media are used to test milk and water for the vibrios.

The El Tor vibrio differs from the true cholera vibrio in that it is hemolytic and gives a positive Voges-Proskauer test. The Celebes strain of El Tor is pathogenic, while the Mecca strain is not (Felsenfeld).

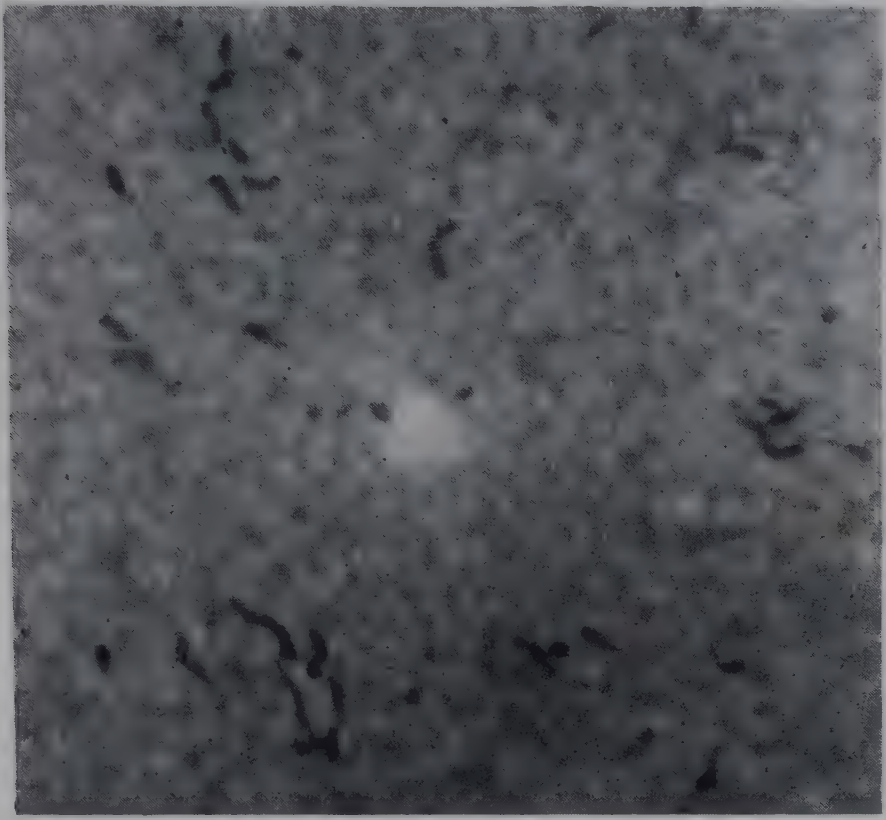

Fig. 382.—*Vibrio comma*, broth culture (×2,000). (Courtesy Dr. Oscar Felsenfeld.)

SEROLOGY

The entire group of cholera vibrios have a common H antigen, while the true cholera vibrios (types Inaba and Ogawa) have a common O antigen, called O-I formerly. Now it is designated as "A" antigen. Formol does not destroy the O antigen of the vibrios.

H + O sera.—Grow the vibrios in infusion broth M-4, pH 8.0. After 24 hours kill by the addition of 0.3% of formol. Inject 1 c.c. doses of this antigen 3 to 4 times at weekly intervals into rabbits.

O sera.—Monovalent sera are obtained by growing the organisms in the presence of antisera and transferring the growth to an agar plate. Wash the growth from the agar with saline and keep in the boiling water bath for 2 hours. Standardize to tube 5 of the nephelometer (page 1477) and inject at weekly intervals 0.1 c.c., 0.2 c.c., 0.5 c.c., 1.0 c.c., 1.0 c.c., 2.0 c.c., and 2.0 c.c., respectively, into rabbits.

Burrows has grouped cholera vibrios according to their "O" antigens. As stated above, the "A" antigen is common to all types. The Ogawa type has the basic antigenic structure A,B . . .; the Inaba A,C . . .; the Hikojima A,B,(C) . . . The most important diagnostic antigens seem to be B and C. Useful and specific sera may be prepared by cross-absorbing Inaba and Ogawa strains. Burrow's work has been confirmed and expanded by Kauffmann, Galut, and others. Pandit and Felsenfeld showed that the "H" antigen of cholera vibrios is not homogenous and cannot, therefore, be used as a means of identifying the genus.

Patient's serum can be used for diagnostic agglutination tests. Use the same technic as suggested for the macroscopic Widal test, using the proper antigen.

Use suspensions of boiled vibrios for the determination of O agglutinins, and 0.2% formolized cultures for the detection of H-O antigens.

The agglutinins are detectable on the fourth day of disease, and usually reach their maximum on the seventh day of the disease.

Paracholera Vibrios

Morphology: Curved rods, often shorter and thicker than *V. comma.* Motile, possessing a polar flagellum.

Stain: Gram-negative.

Colonies: They frequently produce a gray, plumose, moist, glistening growth on agar. They do not coagulate litmus milk. They are found in fowl diarrhea and in stools of man. Their pathogenicity is questioned.

LACTOBACILLUS, LISTERIA, ETC.

Lactobacillus Boas-Oppleri

Morphology: Rods, 1.0 by 6.0 to 8.0 microns, occurring in pairs and in chains. Nonmotile.

Stain: Gram-positive.

Colonies: No growth on agar. Milk agar colonies are small, gray, and granular. Litmus milk is acidified with coagulation. They form acid in dextrose and lactose. They form levorotatory lactic acid in milk. They are found in normal gastric contents, but particularly in gastric carcinoma and other diseases causing stagnation of the stomach contents.

Lactobacillus Bulgaricus

Morphology: Rods, 1.0 by 2.0 microns, often occurring in long chains. Nonmotile.

Stain: Gram-positive, older cultures show gram-negative forms.

Colonies: On whey agar, they form circular to irregular, grayish white filamentous colonies. They acidify litmus milk, with a soft creamy curd in twenty-four hours. They form acid in dextrose, lactose, galactose, levulose, and mannitol. They form 2.7 to 3.7% lactic acid in milk. The lactic acid formed is either inactive or levo-rotatory. They also form a small quantity of volatile acid. They are microaerophilic.

Fig. 383.—*Lactobacillus acidophilus.* (×950.)

Lactobacillus Acidophilus

Morphology: Rods, rather slender, frequently with tapering ends, 4.0 to 5.0 microns in length occurring singly and in short chains. Nonmotile.

Stain: Gram-positive, although older cultures may also show gram-negative forms.

Colonies: Thin, irregular, translucent, fimbriate margin. They form acid in litmus milk, usually with a soft coagulum after forty-eight hours. Acid formed in dextrose, maltose, lactose, sucrose, and raffinose. Some cultures also ferment mannitol. Cultures give off odor of acetic acid.

L-agar (M-14) is recommended for the growth of Lactobacilli.

Listeria Monocytogenes

Morphology: 0.5 micron broad, 1 to 5 microns long, small organisms, occurring as single cells, in V-shaped pairs, or in short chains. Gram-positive. Nonsporulating, noncapsulated, motile with a single flagellum. Pathogenic for mice.

Colonies: Aerobic and facultative anaerobic. Small, transparent, hemolytic colonies on blood agar M-28, M-29. Acid from dextrose, rhamnose, and salicin. Indol, gelatin, and nitrate negative.

Four serologic types are known.

Causes meningitis in man and listerellosis in ruminants. Plating to tryptose agar with 0.05% potassium tellurite greatly facilitates its isolation.*

Bacteroides

Small, slender rods, gram-negative, nonmotile, nonsporeforming. Anaerobic. No proteolytic action; no hemolysis. The genus has not as yet been completely described.

ERYSIPELOTHRIX RHUSIOPATHIAE

Small, slender, nonmotile organisms, appearing in chains which branch freely; often resembling Actinomycetales. They are gram-positive. They grow best under anaerobic conditions, forming alpha-hemolytic, granular colonies. They produce hydrogen sulphide and do not form indol. After adaptation to aerobiosis, the colonies are smooth when grown in oxygen-containing atmosphere.

Dextrose broth with 10% serum is favorable for primary isolation. Subcultures are made on blood agar plates with 1% dextrose.

Inoculation of mice helps to confirm the diagnosis.

PLEUROPNEUMONIA ORGANISMS

These are small, pleomorphic organisms which are often invisible through the ordinary optic microscope. They are gram-negative, and stain well with Wayson's method (S-37).

For primary isolation, thioglycollate broth (M-48) and Norman and Kuhn's broth (M-116) are the media of choice. Since the growth of pleuropneumonia organisms does not cause visible turbidity in all cases, blind daily subcultures are made to further thioglycollate broth tubes and to Norman and Kuhn's plates (M-117). It is necessary to cut out pieces of the solid medium and examine under the microscope to see the colonies.

Frequently such pieces are put into thioglycollate broth for subculture. After several inoculations from broth to broth, visible turbidity will develop.

BARTONELLACEAE

Bartonella Bacilliformis

Morphology: Pleomorphic, coccuslike, ring, oval, rod-shaped (straight, or more or less curved), and granular forms are found. In red blood cells, they form a short chain of cocci or a chain of rods, or they may resemble Chinese letters or a circumflex accent. In endothelial cells lining blood and lymph channels of viscera from fatal Oroya fever, they are sometimes discrete and rod-shaped, but commonly granular or amorphous round masses, distending the cells. In cutaneous nodules, they appear within endothelial cells as rod-shaped organisms or in clusters. Very slowly motile in fresh preparations of blood in Oroya fever. Motile in young cultures. Three to five unipolar flagella. Nonmotile spirals seen in dark-field examination, up to 50 microns in length.

Stain: Gram-negative; reddish-violet with Giemsa and Wright stains. Bipolar staining frequently observed.

Cultivation: They have been grown in Noguchi leptospira medium and in Geiman semisolid and solid media. Growth in Noguchi medium appears in 5 to 15 days, as a nebulous zone 5 to 10 mm. below the surface of the medium. Granular colonies sometimes observed. Tissue cultures have been successful, the organisms developing within the cytoplasm of cells. Beef infusion agar containing cystine, glucose, and rabbit blood have been used, and also chorioallantoic fluid of the developing chick.

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*Gray, M. L., Stafseth, H. J., and Thorp, F., Jr.: J. Bact. 59: 443, 1950.

The general consensus is to classify *Bartonella bacilliformis* as a bacterium. It is the cause of human bartonellosis, Carrión's disease, or Oroya fever.

Human Bartonellosis, Carrión's Disease, Oroya Fever

Distribution.—Human bartonellosis occurs only in certain regions of western South America, from approximately 2 degrees north to 13 degrees south latitude. The disease is observed in two regions in Peru: one, the Pacific watershed; the other, the Marañon watershed. It is seen near the Peruvian border in Ecuador, and in the southwestern part of Colombia, in a large area around the city of Pasto.[*]

Transmission.—Transmission is by the sandfly, Phlebotomus. The mechanism of transmission is through feeding.

The only known reservoir of virus in human bartonellosis is man. Man and the insect vector are the only living beings known to be naturally infected. The incidence of asymptomatic human carriers in infected areas is 10% or higher.

Diagnosis.—The organism must be demonstrated in the peripheral blood. This can be accomplished by examining films, both thin and thick, after staining with Wright or Giemsa stain. When the organisms are not demonstrable in the films, blood cultures must be made. Early in the disease the blood cultures will be positive before the organisms can be found in the blood films.

When the disease is well established, there is irregular fever, rapid and severe anemia, high erythrocytic sedimentation rate, pains in the joints, enlargement of the lymph nodes, and, coupled with history of the environment in which the patient has recently lived, all serve to make the diagnosis.

During the eruptive stage, diagnosis is established by the characteristic appearance of the verrugas, and confirmed by demonstration of the Bartonellae in the lesions. Sections should be fixed in Regaud's fixative and stained with Giemsa stain.

The blood picture is discussed on page 733.

The verruga may resemble an angioma, an angiofibroma, or a botryomycoma,[†] but these tumors are always single and congenital. The subctuaneous nodules, when they have not eroded the skin, may resemble fatty tumors, fibromata, and similar pathologic conditions. However, the coexistence with typical miliary verrugas, the localization near the joints, and the course of the disease furnish the diagnosis. In doubtful cases, bacteriologic and histologic studies of the lesions should be made. In this period blood cultures are often positive.

The disease is not transmissible to the usual laboratory animals. Even monkeys are difficult to infect.

BORRELIA[‡]

Relapsing Fever

The etiological agent of relapsing fever is a spirochete belonging to the genus Borrelia. Many "species" have been described as the causative agent of relapsing fever, including *Borrelia duttonii, Bor. hispanicum, Bor kochii, Bor. novyi, Bor. obermeieri, Bor. recurrentis,* etc. At the present time, it is not definitely known whether these so-called species or races are true, separate taxonomic species or whether they merely represent strains or varieties of one or two species. Morphologically, all of the so-called species and strains are identical. Likewise, the diseases produced are similar clinically. These strains or variants differ from each other principally in their serologic characteristics. It is claimed that in a patient or animal any given strain of Borrelia assumes different serologic characteristics during each relapse.

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[*] For further details, the reader is referred to Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, The C. V. Mosby Company, 1951.

[†] H. Groot, in Clinical Tropical Medicine by Gradwohl, Benitez Soto, and Felsenfeld, St. Louis, 1951, The C. V. Mosby Co.

[‡] Revised from A. A. Packchanian in Clinical Tropical Medicine by Gradwohl, Benitez Soto, and Felsenfeld, St. Louis, 1951, The C. V. Mosby Co.

Morphology: The spirochetes which cause relapsing fever are cylindrical or slightly flattened, with pointed ends, and measure from 8 to 20 microns in length and 0.2 to 0.3 micron in diameter. Each spirochete has about 3 to 7 large, wavy, irregular, inconstant spirals, each spiral having an amplitude of 1.5 microns. These microorganisms are highly motile and divide by transverse fragmentation.

Stain: Borrelia stain readily with Wright, Giemsa, and other common bacteriologic stains.

Cultivation: The spirochete of relapsing fever probably has never been successfully cultured in vitro. Because of their flexibility and small diameter, relapsing fever spirochetes can be passed through a thin-walled Berkefeld filter.

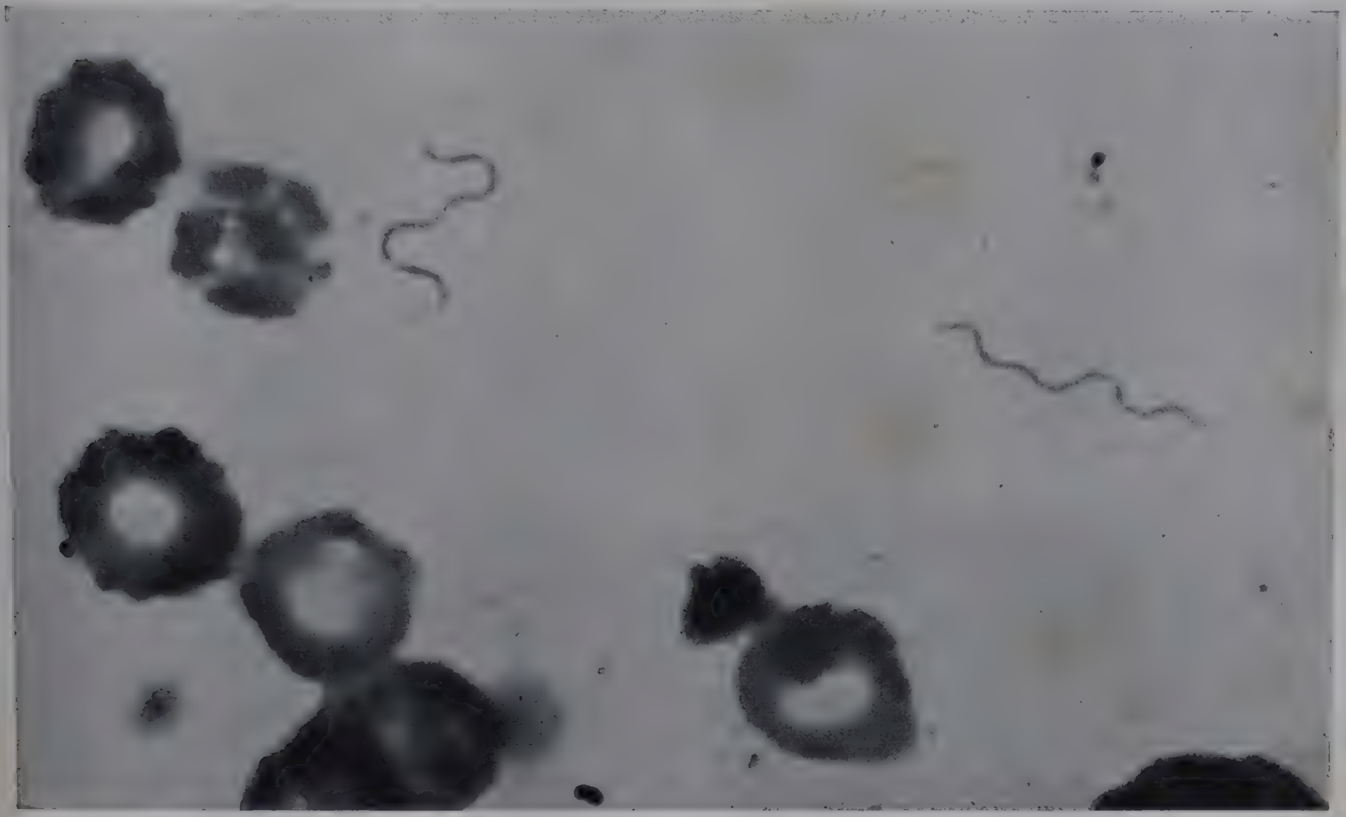

Fig. 384.—Thin blood film showing spirochetes of relapsing fever. Wright stain. (×2,700.) (From Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, The C. V. Mosby Co.)

During the febrile period, the motile spirochetes with lashing movements may be demonstrated in a fresh wet preparation of the patient's blood by dark-field examination, preferably with a high-power, dry objective.

Stained thin or thick blood films are valuable for detection of the microorganisms in the blood.

When the central nervous system is involved, the spirochetes can be demonstrated at times in the cerebrospinal fluid.

Inoculation of laboratory rats with the patient's blood is also a valuable method of diagnosis. Place about 5 to 10 c.c. of the patient's blood, taken during the febrile period, in a sterile test tube containing a few glass beads, and defibrinate by shaking.

Inoculate each of two rats with 1 or 2 c.c. of the defibrinated blood.

From the third to the twelfth day, examine the rat's blood each day for the presence of spirochetes.

The sample of defibrinated blood may be refrigerated for several weeks before inoculation of the rats, if necessary. Defibrinated blood containing Borrelia, stored in an icebox for 2 or 3 months, still retains its power to infect susceptible test animals.

Borrelia have never been cultured successfully in vitro. There are no successful serologic tests.

Distribution of Relapsing Fever.—

Both tick-borne and louse-borne relapsing fevers are widely distributed and found in most parts of the world. The louse-borne disease is found in cold as well as in warmer climates, while the tick-borne relapsing fever occurs chiefly in warmer regions.

Louse-borne relapsing fever appears usually in the form of small or large outbreaks or epidemics, although occasionally sporadic cases are encountered. The infection apparently is not transmitted to man by the bite of the infected louse; however, if the louse is crushed and the material is rubbed into abraded skin as by scratching, infection may take place. It is believed that the spirochetes do not pass through the louse egg.

Louse-borne epidemics are frequently associated with poverty, unhygienic conditions, and troop movements. The disease is often found in close association with louse-borne typhus, and in some cases mixed infections of typhus and relapsing fever coexist. Apparently there is no reservoir host other than man in the louse-borne relapsing fever, and therefore delousing of people and of their clothing is an effective means of limiting the spread of the disease.

Tick-borne relapsing fever occurs usually sporadically. Infected ticks transmit the spirochetes by biting man. The soft ticks which are known to be naturally infected are *Ornithodorus parkeri, O. hermsi, O. rudis, O. talaje, O. turicata, O. erraticus, O. moubata, O. savignyi, O. asperus, O. tartakovski, O. tholozani,* and *O. verrucosus.*

The soft tick remains infected for life and passes the infection through its egg. It therefore seems that Ornithodorus is the principal reservoir host of *Borrelia recurrentis,* although mammals such as monkeys, chipmunks, prairie dogs, rats, and squirrels may acquire the infection from ticks and serve as reservoirs for a shorter period.

Crab lice (*Phthirus pubis*) and bedbugs (*Cimex lectularius*) apparently play no role in transmitting relapsing fever.

The spirochetes may be demonstrated in the spleen, brain, and other organs.

Laboratory Findings.—

During the febrile period, albuminuria is usually present. The leukocyte count is elevated to about 10,000 to 20,000 while there is fever. The Wassermann reaction is said to be positive in a small percentage of cases.

TREPONEMA

Treponema Pallidum

(Spirochaeta Pallida)

Morphology: Cylindrical, 0.25 to 0.3 by 6 to 14 microns, with pointed ends. Spiral amplitude, 1.0 micron, regular. Spiral depth, 0.5 to 1.0 micron, very constant. Waves, one or more slight, undulating curves may be present. Axial filament doubtful. The whole seems to consist of a spirally wound axial filament. Chambered structure absent. Membrane doubtful, if there is one present, it must be flexible. Crista absent. Terminal finely spiral filament present, and easily seen in cultures. Flagella absent. Highly motile end portion absent. Division transverse or possibly also longitudinal.

Stain: Diagnosis is usually made by means of the dark-field microscope, where the organisms may be observed alive. In this way they can be identified by their movement. They stain pink with the Giemsa stain. They are disintegrated in bile salts in the proportion of 1:10. The Krajian method of staining is recommended. (See pages 2138 and 2141.)

Cultivation: They have been cultivated under strict anaerobic conditions in serum-water containing fresh tissue.

T. pallidum is the microbic agent that causes syphilis.

Treponema Microdentium

Morphology: They are less than 0.25 micron in thickness in the center, and taper toward each extremity, which is pointed. The length varies with age, although they may reach 8 microns with 14 curves. Sometimes a long, thin flagella-like projection is observed at each extremity.

Cultivation: Strict anaerobes, cultivated on serum-water with fresh tissue. Nonpathogenic. They are normal inhabitants of the oral cavity. They may be stained with the Giemsa or the Krajian stain.

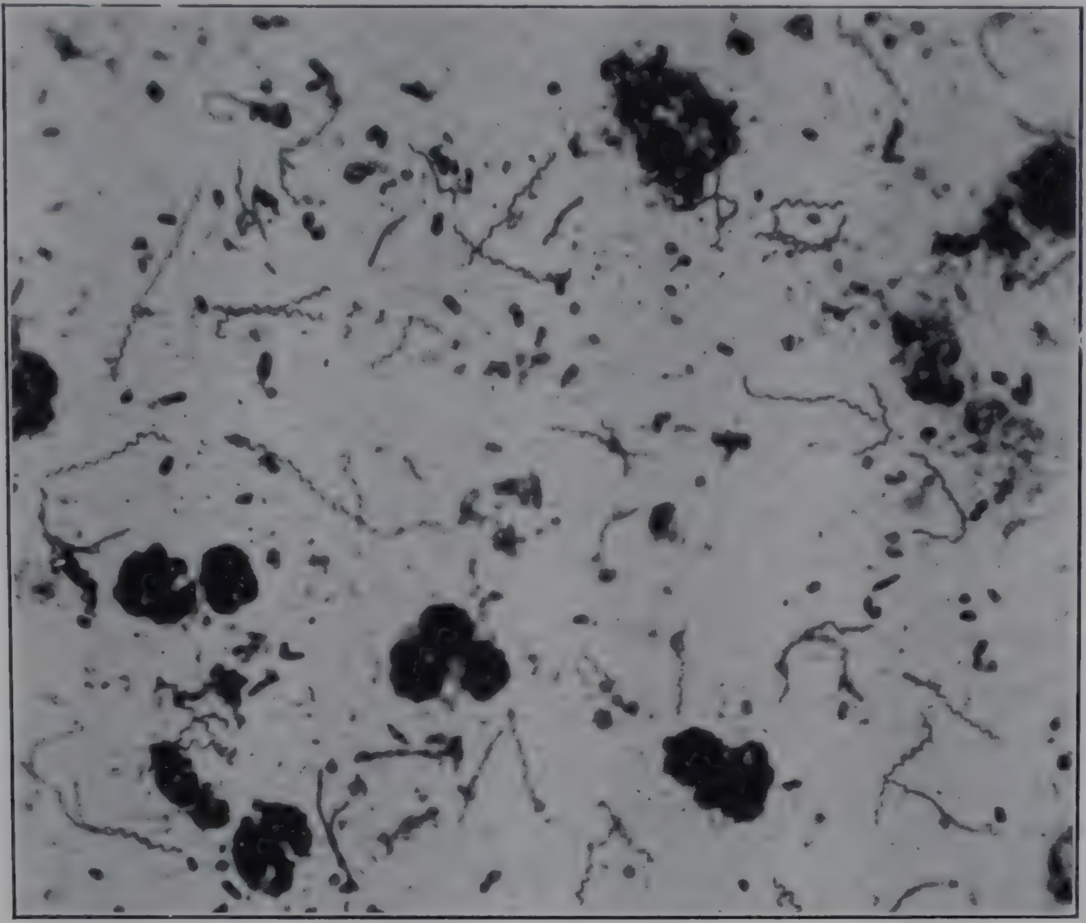

Fig. 385.—*Treponema pallidum* in a biopsy specimen of penile sore. Stained by the Krajian 20-minute method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, St. Louis, 1952, The C. V. Mosby Co., by permission.)

Treponema Macrodentium

Morphology: Spirals with abruptly tapering extremities. They are 0.7 to 1.0 by 3.0 to 8 microns, with 2 to 8 curves.

Stain: They may be stained with the Giemsa or the Krajian stain.

Cultivation: They are obligate anaerobes, and are cultivated in serum-water containing fresh tissue. They are normal inhabitants of the oral cavity. Nonpathogenic.

Treponema Carateum*

(Treponema Herejoni)

Morphology: In dark-field illumination, they appear as brilliant cylindrical filaments, coiled along the long axis. The coils are tight, regular, and rigid, especially in the center. At times, these coils relax and separate, to return to their original position. Length, 7 to 22 microns; thickness, 0.2 to 0.3 micron; depth of spirals, 0.8 to 1 micron; number of spirals, 7, 15, to 29. Very highly motile, turning rapidly about the long axis. Bipolar movements of progression. When stationary, in addition to the spinning or drilling movements, there are movements of torsion and flexion, arching, and assuming shapes of letters O, P, and S. They have undulant and creeping movements, so that they move in and out of focus. Movement is lost in 1 to 6 hours. They cause mal del pinto, or pinta.

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*Revised from F. León-Blanco in Clinical Tropical Medicine by Gradwohl, Benitez Soto, and Felsenfeld, St. Louis, 1951, The C. V. Mosby Co.

Stain: They stain well with Giemsa stain and especially well with the Krajian spirochete stain.

Cultivation: They have not been successfully cultivated.

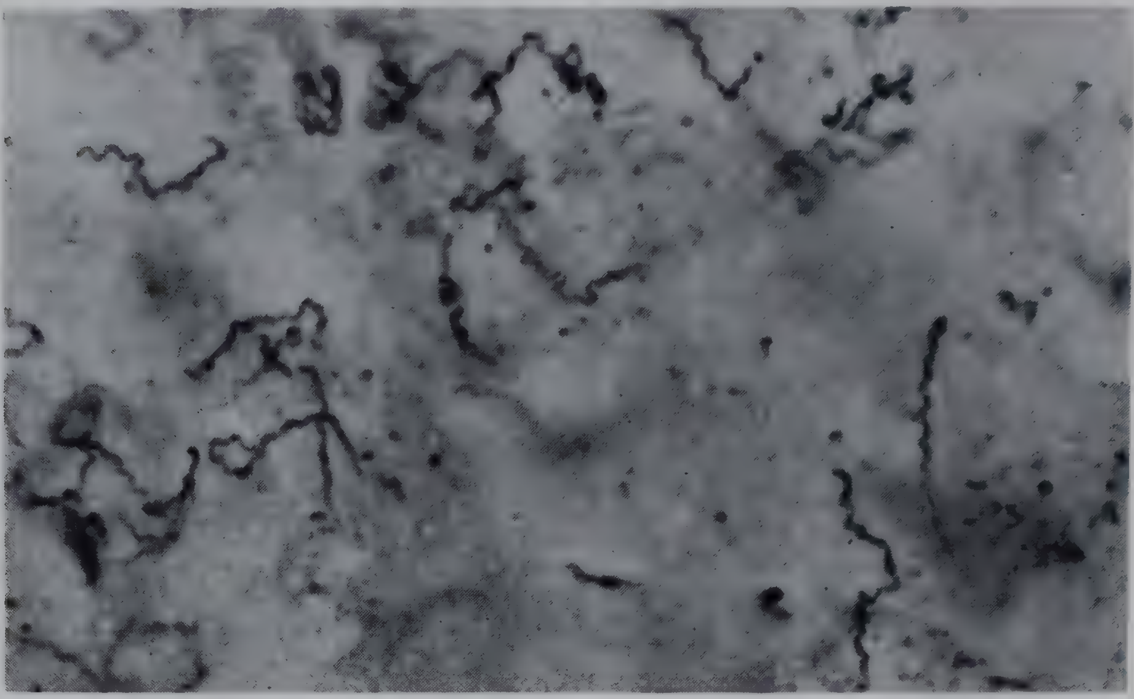

Fig. 386.—*Treponema carateum* in skin in a case of mal del pinto. Service of Dr. Pequeño. (Stained with Krajian spirochete stain; ×950; reduced ⅕.)

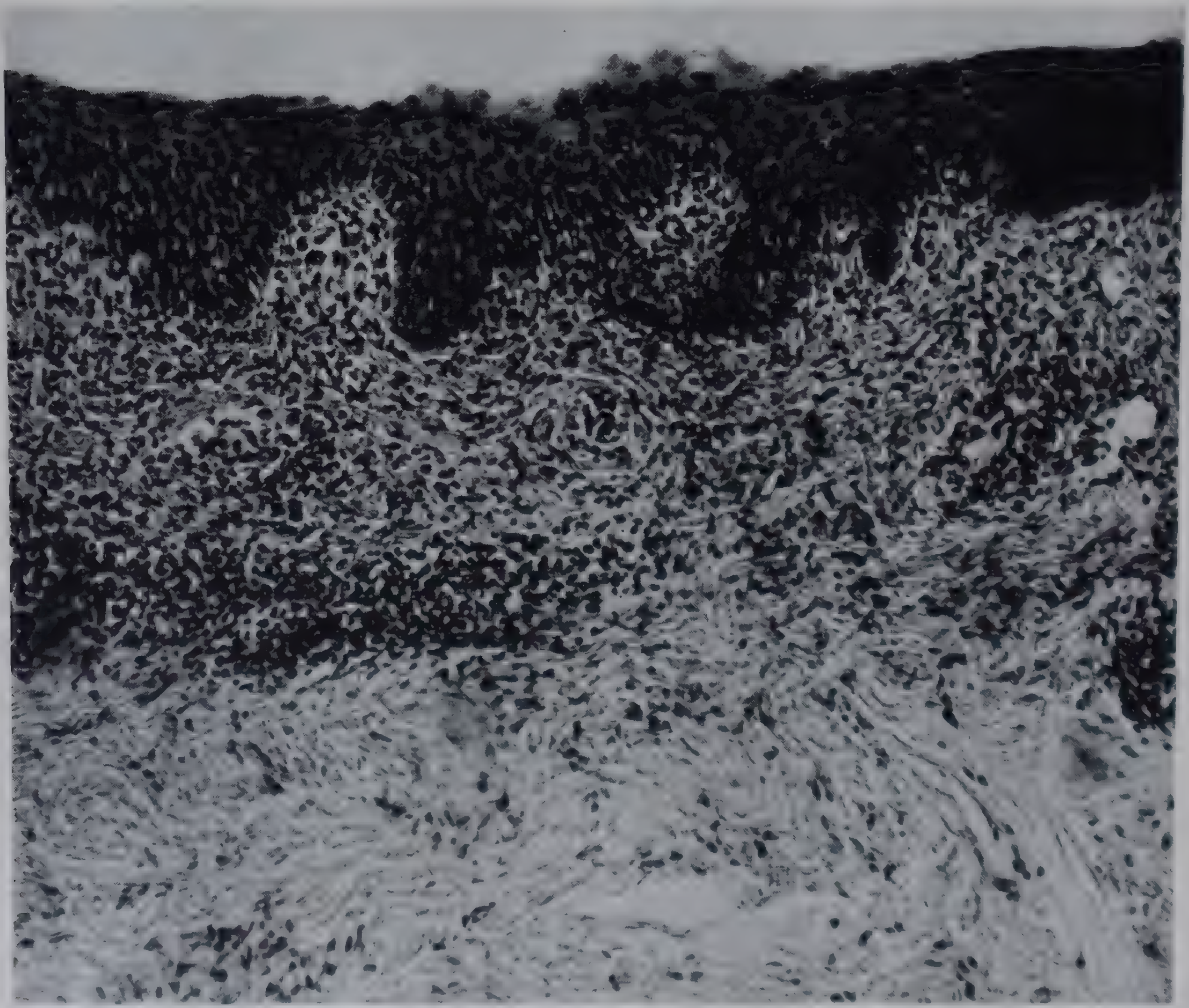

Fig. 387.—Section of skin in mal del pinto. (Service of Dr. R. B. H. Gradwohl.)

According to León-Blanco, the distribution of the treponemata in cutaneous lesions in man varies for the initial lesions of mal del pinto (carate) and pintids, and also for lesions of the later phase. In the initial lesions and in the pintids, treponemata are particularly abundant in the epidermis and in the intercellular spaces of the rete mucosum, especially in the acanthotic interpapillary crests, as well as in the epithelial portion of the pilosebaceous follicles. In the dermis they are localized especially in the portion occupied by the inflammatory infiltration, and they are abundant near the blood vessels. It is not exceptional to see them as they traverse the walls of these vessels. In the later phases, they have been seen only in the rete mucosum and in the epithelial portion of the pilosebaceous follicles.

Distribution of Mal del Pinto.—

Mal del pinto (pinta) is endemic in rural or suburban areas, stopping at the outskirts of large cities, particularly in countries with a high epidemic index. Where the disease is sporadic, it can be found in large urban centers.

In the intertropical regions of the Americas, the disease is most widely distributed in villages situated in the valleys of large rivers, at an altitude which varies from sea level to 2,570 meters. In some areas in this zone, incidence reaches 50 to 90%, while in localities a short distance from these zones, only 1 to 2% of the population is affected. Apparently there is no relationship between the disease and geographic conditions. Infection is more frequently seen among inhabitants of villages and small towns situated between mountainous zones and the sea than among residents of towns which lie near the coast. In coastal zones, rural towns and villages show a higher index of infection than less urbanized areas.

In villages with high incidence of the disease, it is found in family foci, although intimate contact with pintous patients is not the sole prerequisite for infection. It is frequently observed that in marriages of one to twenty-five years, one marital partner may be ill with the disease while the other has remained healthy (León-Blanco).

Where the infection is sporadic, pinta is not transmitted to those who live with infected individuals.

When members of the Caucasian race live under conditions similar to those of Indians, Negroes, and mestizos, they are as easily infected. There is no special susceptibility to pinta on the part of certain races when the mode of life is identical. Sex has no bearing on incidence of the disease. Pinta can occur at any age, although the initial lesions are most frequently seen between the ages of 5 and 20 years.

Diagnosis.—Examination for treponemata in cutaneous lesions and in glands may be carried out by silver impregnation of biopsy material. We recommend the method of Krajian. The dark-field examination may be made of serous matter obtained by puncture of the glands or by erosion and expression of cutaneous lesions. This method is the most rapid and the easiest.

Technic.—With a bistoury or curette, make a very superficial abrasion in the skin of the suspected lesion.

Apply pressure with a pair of forceps. A few drops of serous matter will be expressed from the lesion.

If the first drops contain many red blood cells, discard until a clear drop is obtained.

Mount the drop on a slide under a cover glass, and examine by means of the dark-field microscope.

If desired, make a smear and stain by the Krajian spirochete method.

Treponema Pertenue*

Morphology: 12 to 20 microns long, with 8 to 20 small, broad turns in the spiral. Morphologically indistinguishable from *Treponema pallidum*. Motility by translation in the direction of the long axis, a rotary movement of the spirals, giving the impression of a corkscrew turning on its own axis, and a waving or twisting movement from side to side.

Stain: They require special methods of staining. They stain easily with the Krajian spirochete stain.

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*Revised from A. J. Sheldon in Clinical Tropical Medicine by Gradwohl, Benitez Soto, and Felsenfeld, St. Louis, 1951, The C. V. Mosby Co.

Cultivation: Cultivation is difficult, if not impossible.

They cause yaws or frambesia.

The organisms are readily killed by drying and cannot withstand exposure to air and light more than 24 hours in a moist medium.

The spirochetes are present in large numbers in primary and secondary lesions and have been demonstrated in lymph glands, spleen, and bone marrow. They have not been found in the blood stream, but Castellani successfully infected apes by injecting blood from frambesia patients. Further evidence of their presence in the blood stream is the dissemination of the secondary lesions over the entire body. Organisms have never been demonstrated in deep organs other than those mentioned above, nor in the nodular, ulcerative, scaling, or gummatous lesions of the tertiary stage of the disease. Monkeys and rabbits, as well as apes, can be experimentally infected with frambesia. Hamsters are also susceptible.

Transmission (Yaws or Frambesia).—

Treponema pertenue is readily transmitted from infected persons to others by direct contact. Indirect contact with contaminated clothing, bedding, sleeping mats, utensils, furniture, soil, etc., is probably a less important means of spreading the disease because the organisms are quite fragile. Mechanical transmission by the "gnat" fly, *Hippelates papillipes*, is possible within seven hours after feeding on open frambesia lesions, by regurgitation of ingested material containing spirochetes. Venereal transmission does occur but is only incidental. Autoinfection may occur during the early course of the disease. Lesions frequently spread locally and to opposing contiguous surfaces, particularly the mouth, anus, and vagina. It is not believed that congenital infection occurs, although interstitial keratitis has been found among frambesia patients in Samoa where syphilis does not exist among the native population.

Trauma plays an important role in the transmission of frambesia. It seems likely that the organisms must come in contact with an abrasion in the skin in order to initiate successful infection. It is questionable whether or not they can penetrate the intact skin. It is generally accepted that the location of the primary lesion is also the site of infection. Primary lesions occur most frequently on exposed regions of the body which are most likely to be traumatized: the feet and legs. Crowded, unhygienic living conditions foster spread of infection in family groups so that frequently all of the children and sometimes the adults in a family develop lesions.

Persons of all ages are susceptible to infection with this organism, but the disease occurs most commonly in children.

Immunity.—

It is believed that one attack of yaws confers protection against another in regions where the disease is endemic, the immunity developing slowly and over a period of years. This immunity is not always complete or constant.

Distribution.—

The disease is seen mostly among Negroes, although it has been found in Polynesians, Melanesians, yellow-skinned races, Indians, and European races. A few cases have been found among the Caucasoids in the United States following residence in or visits to tropical areas where frambesia is endemic. If racial immunity exists, it plays a secondary role to unhygienic environmental conditions in the dissemination of frambesia.

Diagnosis.—Yaws must be differentiated from syphilis, particularly in the tertiary stages. Little aid can be obtained from the laboratory, since the etiological agents are indistinguishable, and specific serologic tests are reactive in both diseases. Spirochetes can be readily demonstrated by dark-field microscopic examination of serum from primary and secondary frambesia lesions after removal of the crusts. The usual serologic tests for syphilis, complement fixation, flocculation, and other specific tests are reactive in over 99% of patients with early frambesia. The tests become reactive two to three weeks after the appearance of the primary lesion. The titer increases rapidly and becomes strongly reactive during the fifth or sixth week. The tests remain reactive for months or years, occasionally becoming nonreactive in the tertiary stage.

LEPTOSPIRA*

Morphology: Leptospirae belong to the family Spirochaetaceae. They are characterized by a flexible threadlike structure consisting of a large number of regular spiral coils. The amplitude of each coil is approximately 0.5 micron. The length of the entire Leptospira varies between 8 and 12 microns. Degenerative forms may reach a length of 40 microns. The width is constant and approximately 0.25 micron.

Multiplication is by transverse fission, which may be seen under the microscope.

Leptospiras are visible under the dark-field microscope. They look like strings of very closely packed small beads. The ends are hooked, the center part rather rigid, and they move only slowly through the field, with rapid rotation on the long axis. In viscid media they seem to have some traction and acquire some speed, the middle part appears flexible, the movement becoming snakelike. The ends at times exhibit a whipping motion.

Do not confuse them with so-called ''pseudoleptospiras,'' which are threadlike fibrin structures expelled from blood cells in blood specimens; these show Brownian movement and movement caused by currents in the liquid, and are often mistaken for true leptospiras.

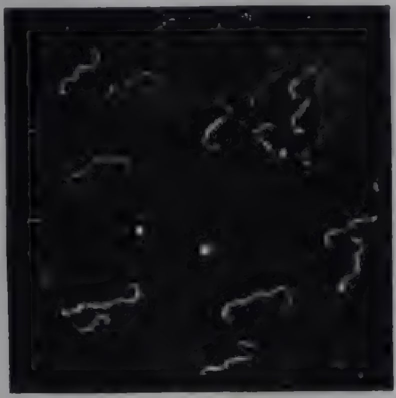

Fig. 388.—*Leptospira icterohaemorrhagiae* (×950). (Kolle-Wassermann: Handbuch der pathologischen Mikro-organismen, Band VII, 1, Table III.)

Staining: Leptospiras are not stained with simple dyes like gentian violet. They are stained readily by the Giemsa method in blood films, and in tissue sections by the Krajian 20-minute method. The primary coils are invisible with the usual fixatives.

To stain with Giemsa stain, use a strong solution, with faintly alkaline water, and stain at 37° C. for 30 minutes. Pour off, and re-stain with dilute Giemsa at 37° C. for another 30-minute period. Wash and examine. Victoria-blue may also give good results.

Take care that the slides are clean and fat-free. Wash the stain off carefully by allowing the water to drip on the stain-covered glass, and rinsing the precipitate with the water. Leptospiras stain violet, and the separate coils can be seen distinctly.

In tissues, the method of Krajian for staining spirochetes is recommended. See page 2138.

Leptospiras are able to pass through the pores of various filters. Their great flexibility, their extreme thinness, and their actively boring movements permit them to pass through the pores.

Cultivation: Leptospiras have no complicated growth requirements, but will grow in simple solutions as follows.

Buffered Peptone Solution

Peptone Difco or BBL	0.1%
Phosphate buffer, pH 7.2	10 %
Distilled water	1,000 c.c.

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*From A. C. Ruys in Clinical Tropical Medicine by Gradwohl, Benitez Soto, and Felsenfeld, St. Louis, 1951, The C. V. Mosby Co.

Sterilize in an autoclave at 15 pounds pressure for 30 minutes.

Usually an enriched medium is preferred, since the leptospiras grow better in such media.

Vervoort Medium

(Modified by Wolff and Schüffner)

Peptone Difco or BBL	1 gm.
Ringer's solution	200 c.c.
Sörensen's phosphate buffer, pH 7.2	100 c.c.
Tap water	1,000 c.c.

Boil until the phosphates have been precipitated and filter after cooling. Distribute into small tubes.

Sterilize at 100° C. The pH is 6.9 to 7.0.

Add 10% sterile rabbit serum and inactivate at 56° C. for 30 minutes. Some rabbit sera agglutinate the leptospiras and cannot be used in the medium, so that each rabbit serum must be previously tested.

Korthof Medium

Peptone Difco or BBL	400 mg.
Sodium chloride	700 mg.
Sodium bicarbonate	10 mg.
Potassium chloride	20 mg.
Calcium chloride	20 mg.
Monobasic potassium phosphate (KH_2PO_4)	90 mg.
Dibasic sodium phosphate ($Na_2HPO_4 . 2H_2O$)	480 mg.
Distilled water	500 c.c.

Heat the mixture for 20 minutes at 100° C. Cool and filter.

Sterilize at 100° C.

Add 8% of sterile fresh rabbit serum.

Tube the mixture and inactivate at 56° C. for 1 hour.

Noguchi Medium

Rabbit serum	1.5 parts
Ringer's solution	4.5 parts
2% agar	1.0 part

Paraffin oil to cover the surface.

Noguchi used this medium also in combination with a fluid medium consisting of 1.5 parts of rabbit serum and 4.5 parts of Ringer's solution poured over the solid medium, the whole covered with paraffin oil. A drop of rabbit hemoglobin—1 part of defibrinated rabbit blood and 3 parts of distilled water—may be added to improve the results.

Savino Medium

Monobasic potassium phosphate (KH_2PO_4)	2 gm.
Sodium chloride	3 gm.
Magnesium sulphate	0.05 gm.
Calcium chloride	0.01 gm.
Distilled water	1,000 c.c.

Add, as activator, 0.1% of yeast extract
0.5% of caseine hydrolysate
and 0.005% of hematin.

Adjust the reaction to pH 7.1 with hydrochloric acid.

Sterilize at 115° C. for 15 minutes.

Add 10% sheep serum sterilized by filtration.

Leptospiras must be cultivated under aerobic conditions. The optimum temperature for rapid growth is 37° C., but lower temperatures are necessary to keep the cultures alive. Incubation is mostly between 28° and 32° C.

To obtain rich cultures, it is necessary to seed with large amounts of material. For subculturing, nearly 0.5 c.c. of the old culture is transplanted into the fresh medium.

Viability and Resistance: Leptospiras show remarkable viability in cultures. Cultures in Vervoort's medium kept in the dark at room temperature without subculturing sometimes remain alive for years. In the semisolid medium of Noguchi, a thick layer of leptospiras grows 10 to 12 mm. below the surface, and these organisms may remain fully virulent for years.

Leptospiras are very sensitive to acid reaction. They are killed very quickly in acid urine. To isolate them from urine, it is advisable to alkalinize the urine by keeping the patient on appropriate diet for some days before collecting the specimen. They are not very sensitive to salts. They may remain alive in sea water for several hours, but disappear more quickly from salt and brackish water than from fresh water.

They are killed at 50° to 55° C. in 30 minutes. They tolerate low temperatures well. They can be kept alive in tissues for several months when frozen at –20° C. In culture fluid at this temperature, however, they survive no more than five days. They cannot be preserved by lyophilization.

They have little resistance against the various disinfectants. Bile kills them quickly. They are not susceptible to sulfa drugs. Of the antibiotics, penicillin is active; it has been used successfully in animal experiments. Good results have been reported in a few patients.

Antigenic Structure

Soon after the discovery of leptospiras as the etiological agent of various infections, the question arose as to whether those strains which differ serologically are independent species or whether they are mere variants of the same species. This problem has not been solved to date, and both theories have their adherents. The first strains recognized as differing from the classic Weil strains were *L. hebdomadis, L. akiyama* A in Japan, and *L. pyrogenes* in Sumatra, all of which cause an acute febrile disease, but mostly without jaundice, and with a much lower death rate than Weil's disease.

When still more serologically different strains had been isolated, a more elaborate study of the antigenic structure became necessary, especially since the various strains cause almost the same clinical picture in man, the differences being unimportant and only in degree. In addition, the virulence for animals is not a reliable means of differentiation either.

Antisera from patients or sera prepared by injection of cultures into rabbits agglutinate cultures of leptospiras often in a high titer.

Because of the faint visibility of the leptospiras in ordinary light, the **agglutination** is poorly visible macroscopically. Pot[1] reported that concentrated Formalin-killed cultures may show agglutination visible to the naked eye, but unfortunately with these antigens the titers are lower and a much greater quantity of cultures is needed.

This method and others based on the same principle have found only few followers, so that the serologic tests must be controlled by the dark-field examination. Serum dilutions made with a buffer solution are mixed with equal quantities of a rich culture and kept at room temperature. After two hours, the results can be read provisionally, but the titer is not determined until the next day.

In the lower dilutions of a strongly active serum, agglutination is clearly visible; in the higher dilutions, lysis complicates the picture. The conglomerates of agglutinated leptospiras begin to show signs of lysis, until, at the end, only shapeless balls can be found, and finally nothing is seen except a few freely floating single leptospiras. The titer end point is the last dilution of the serum which shows some difference in number with the control.

Attention must be called to the fact that sometimes ''nests'' of leptospiras grow in rich cultures. These may resemble agglutinated leptospiras. In the ''nests,'' however, each leptospira is entire and actively moving; in agglutinated conglomerates, the leptospiras have a damaged appearance and the movements finally cease.

To avoid the danger of working with living leptospiras and to be able to have a stock of antigen ready at hand, some laboratories use Formalin-killed cultures. It is necessary to use chemically pure Formalin and no more than 0.5%. Impurities may cause nonspecific acid agglutination.[2] When dead cultures are used, however, the lysis is absent and agglutination becomes manifest in much higher dilutions than with living cultures, where this phenomenon has been obscured by the lysis. The titer of the sera tested with killed antigens is on the whole somewhat lower than when the titer of lysis is determined with living ones.[3] The results with these antigens are less specific than with living organisms. The Formalin antigens can be kept for a limited time only, because clumping begins sooner or later, probably caused by formic acid originating from the Formalin under the influence of light.

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[1] Lancet 1: 1290, 1936.

Complement fixation tests have also been used in serologic work, but they are less specific and have practically been abandoned. To determine the antigenic structure of a strain, monovalent rabbit sera are used, which should have a titer of not less than 10,000. They agglutinate and lyse strains of the same species to the same titer or sometimes less, the lowest being one-tenth of the titer.

Other species may show a strong co-reaction, but they never reach more than one-third of the titer.[4, 5]

Some species are serologically closely related (*L. canicola-L. icterohaemorrhagiae*); others are independent (*L. bataviae*). For an exact diagnosis, however, agglutinin absorption tests are necessary, especially in those cases where co-reactions are strong. The absorption test first described by Ruys and Schüffner[6] and improved by Schüffner and Bohlander[7] is made as follows:

Agglutinin Absorption Test

Use a well-grown culture in Vervoort's medium, killed with 0.5% Formalin. Centrifuge 100 c.c. of this culture for 20 minutes at 10,000 r.p.m.

Pipette off the supernatant fluid.

Mix the remaining 0.45 c.c. of concentrated cultures with 0.05 c.c. of the serum diluted to a titer of 1:3,000.

The antibodies are fixed within 4 hours, but the mixture can be left overnight at room temperature.

Centrifuge at 10,000 r.p.m. for 5 minutes, and use the supernatant fluid for agglutination tests. Van Riel[8] prefers to perform the absorption with living cultures.

It is difficult to absorb a serum with many strains because a leptospira culture cannot be entirely deposited at the bottom of a tube, and some fluid remains in the tube. The serum is diluted with the remaining culture fluid, and it might become too greatly diluted if more strains were used. We have no absolutely monovalent sera such as we use in our bacteriologic serologic work.

Strains of the same species remove antibodies from a serum, strains of a related one only those belonging to the strain itself. The clear-cut results obtained with this method allow of an exact identification. Several species are now recognized as serologic and biologic entities in which there is correlation with a distinct epidemiologic behavior.

However, strains considered to be of the same species may show minor serologic differences. Borg Petersen,[9] whose work was confirmed by Gispen and Schüffner,[10] demonstrated that the classical Weil strains fall into two groups, one complete with the antigens A and B, the other incomplete with only A. Both behave alike pathologically and epidemiologically, so that they may be considered as two biotypes of the same species. Probably the same relation exists between *rachmat*, incomplete, and *akiyami* A, complete biotype. Van Riel found the same relation between an Indonesian strain (Benjamin) and an African strain

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[2] Zuelzer: Zentralbl. f. Bakt. (Abt. 1) **108**: 330, 1932.

[3] Walch-Sorgdrager, Schuffner and Bohlander: Ztschr. f. Immunitätsforsch, u. exper. Therap. **92**: 35, 1938.

[4] Walch-Sorgdrager: Bull. Health Org. League of Nations **8**: 151, 1938.

[5] Walch-Sorgdrager and Bohlander: Ant. v. Leeuwenhoik **5**: 100, 1939.

[6] Nederl. tijdschr. v. geneesk. **78**: 3110, 1934.

[7] Zentralbl. f. Bakt. (Abt. 1) **144**: 434, 1939.

[8] Ann. Soc. Belge Med. Trop. **26**: 197, 1946.

[9] Acta Convent. tertii de trop. atque malar. morbis **1**: 396, 1938.

[10] Zentralbl. f. Bakt. (Abt. 1) **144**: 427, 1939.

TABLE 161.—IMPORTANT LEPTOSPIRA STRAINS*

SPECIES	GEOGRAPHY	HUMAN DISEASE	SEVERITY USUALLY	PRINCIPAL ANIMAL HOST	ADDITIONAL ANIMAL HOST(S)	NOTE
L. icterohaemorrhagiae	World-wide	Weil's disease	Severe	*R. norvegicus*	Other rats, mice, dog, fox, sheep, pig, horse	Biotypes AB A and *naami*
L. canicola	Widespread	Canicola fever	Mild	Dog	Horse, cow, pig	
L. pyrogenes	USA, S-E Asia	Febrile spirochetosis	Mild	*R. brevicaudatus*		*L. salinem*
L. australis B	S. Europe, Japan, Australia	Swamp fever, cane fever	Mild	*R. culmorum*	*R. conatus*	May be identical with *L. pyrogenes*
L. ballum	USA, C Europe	(Leptospirosis)	Mild	*M. musculus albus*	Other rodents, horse, cow, dog	
L. pomona	Widespread	Swineherd's disease	Mild	Pig	Cattle, horse	*L. suis*, *L. australis* C possible subtypes
L. hebdomadis	USA, Japan, S-E Asia	Seven-day fever	Mild	*Microtus montebelloi*	Cattle, dog	*L. wolffi* is subtype
L. sejro	USA, C-S Europe, S-E Asia	Sejrø fever	Mild	*M. spicilegus* *Apodemus sylvaticus*	Other rodents, horse, dog, cattle	
L. saxkoebing	C Europe	(Leptospirosis)	Mild	*A. flavicellis* *A. sylvaticus*	Cattle	
L. medanensis	S-E Asia, Australia	(Leptospirosis)	Mild	?		
L. grippotyphosa	Widespread	Swamp or mud fever Harvest fever Infectious jaundice	Mild	*Microtus arvalis*	Other rodents, cattle	*L. geffeni*, *L. bovis* and *L. andaman* B may be subtypes
L. autumnalis	USA, Japan, S-E Asia	Autumnalis fever Fort Bragg fever Pretibial fever	Mild	*R. brevicaudatus* *A. speciosus*	*M. montebelloi*	Serologic biotypes are AB (*akiyama* A), A (*rachmat* incomplete), Bangkiang
L. bataviae	Widespread	Rice-field fever Water fever Indonesian Weil's disease	Severe	*R. norvegicus* *M.m. soricinus*	Other rodents, pig, cat, dog	*L. oryzeti* identical *L. mitis* closely related
L. mitis	C-S Europe, S America	Swineherd's disease	Mild	Pig	Horse, cattle	*L. hyos* identical
L. australis A	Widespread	Swamp and cane fever	Mild	*R. culmorum*	Other rats, dog, horse, cattle	*L. baillico* identical
L. javanica	Widespread	(Leptospirosis)	Mild	*R. conocolor* *R. brevicaudatus*	Other rats, cat	*L. poi* identical

*Compiled after Ruys, Wolff, and Broom.

(Mukingilwa), neither of which has been fully tested with all other sera. According to van Riel, *L. canicola* also has the two types mentioned here. Table 161 is a list of strains examined with agglutinin absorption tests.

There are a number of other strains which have been more or less exhaustively examined: *L. bovis, L. vituloreum, L. icterohaemoglobinuriae, L. schüffneri, L. mukingliwa, L. andaman* A, the strains H and H.C., Veldrat, Semarang 173, Djasiman, and many others. Our knowledge of either the serology or the epidemiology of these organisms, however, is still too incomplete to include in Table 161.

Variability

So far as I (A. C. R.) know, no variation in serologic behavior has ever been observed. The old strains from the Amsterdam collection have all remained stable. No conclusive animal experiments have ever shown a transition of one type into another. The experiments of Uhlenhuth and Zuelzer[11] and of Baermann and Zuelzer,[12] who considered the various strains to be all variants of one classical species, are open to criticism.

Van Riel,[8] however, basing his conclusions on his experiments in Africa with a number of strains but not with all types now available, defends the thesis that intermediate types exist and that there are transitions from one type to another. He emphasizes that strains which belong serologically to *L. grippotyphosa* in Central Africa cause a disease with frequent jaundice and a high death rate. However, as long as no exhaustive study of the epidemiology has been made, the picture is not complete and conclusions are premature.

DEMONSTRATION AND ISOLATION OF LEPTOSPIRAS

In all leptospiral infections the microorganisms circulate in the blood during the first week of illness. After the first ten days, positive blood cultures are very rare. Bone marrow has been found positive even on the twentieth day.[13] In the latter phases of the disease the leptospiras may be excreted with the urine.

Sometimes the spinal fluid contains leptospiras in incipient stages. At autopsy, both the liver and the kidneys may contain leptospiras.

Leptospiras in the Blood

Blood films stained with Giemsa stain rarely show leptospiras. Ordinary dark-field examination of blood also rarely gives a positive result, and many so-called positive findings have been caused by pseudoleptospiras mistaken for actual leptospiras. With the centrifugal method, however, leptospiras may be demonstrated in the blood in some cases during the first days of the illness.[14]

Mix 1 part of 1% sodium oxalate solution in phosphate buffer pH 8.1 with 9 parts of blood.

Centrifuge 15 minutes at 1,500 r.p.m.

Examine the clear plasma under the dark-field microscope with low magnification. If no leptospiras are found, centrifuge the remainder of the plasma for 20 minutes at 10,000 r.p.m. The sediment might show the leptospiras.

Blood collected in Liquoid Roche can be used for dark-field examination after the first centrifugation, and sediment can be obtained from the plasma.[15]

Liquoid Roche: 15 to 20 c.c. blood in 4 c.c. of a 1% polyanethol sodium sulphonic acid solution in saline = Liquoid.

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[11] Klin. Wchnschr. **1**: 2124, 1922.

[12] Centralbl. f. Bakt. (Abt. 1) **105**: 345, 1928.

[13] Dreyfuss and Montefiore: Compt. rend. Soc. de biol. **131**: 73, 1939.

[14] Ruys: Nederl. tijdschr. v. geneesk. **77**: 3654, 1933.

[15] van Thiel: The Leptospirosis, Leiden, 1948, Universitaire Pers.

This rapid method, which permits an early diagnosis within half an hour after the sample has been taken, is not absolutely reliable. At times microscopically negative blood yields positive cultures or animal inoculations. All three methods should be used to demonstrate the leptospiras. The culture is the most reliable, but often positive results are obtained only after one and one-half to four weeks. If the material is contaminated, the addition of 400 mg. of sulfonamide to the medium may give a pure culture of leptospiras.[16]

To demonstrate the leptospiras by means of test animals, young guinea pigs, 150 to 200 gm., should be used, preferably also young hamsters, because these animals are sensitive to leptospiras (*L. canicola*) which are only slightly pathogenic for guinea pigs.[17]

The inoculation should be made into the peritoneal cavity, with 0.5 c.c. of blood. Daily examinations from the third day on may show leptospiras even in those cases in which the strain does not cause the animal to become sick. If the animal develops fever, the blood is usually positive, and pure cultures may be obtained by inoculation of the blood, obtained by puncture of the heart, into suitable media. Guinea pigs dying of leptospirosis show hemorrhages and mostly a severe jaundice. At autopsy the liver and kidneys sometimes contain leptospiras in abundance; in other cases very few or none are found. The adrenals are always positive.

White mice are also used; they are susceptible to many strains of leptospiras, but one must bear in mind that white mice may also be spontaneously infected, as first described by Bessemans and Thiry[18] and later by Schüffner (not published). Ruys and associates[19] found two colonies of white mice to be heavily infected with *L. ballum* which were excreted in large quantities. One must be sure that the colony of mice in use is free from spontaneous infection. Bessemans and co-workers[20] demonstrated that some strains of *L. icterohaemorrhagiae* kill the mice quickly and that others cause practically no symptoms except a constant urinary excretion of leptospiras virulent for guinea pigs.

Leptospiras in Urine

Leptospiras may appear in the urine from the beginning of the second week of the illness on. Direct examination in the dark field sometimes shows the leptospiras in great numbers. If the urine is acid, most of them will be dead and will show the typical rigid appearance. Passage through a susceptible animal (guinea pig, hamster) is necessary to isolate them from the urine.

The patient should be given an alkalinizing diet a few days before the specimen is to be collected, to prevent the damage of acid urine. The simplest way to obtain a positive result is to take the second portion of urine of the day and to inoculate the animals intraperitoneally with 1 c.c. of the freshly excreted urine at the bedside of the patient. Urine sent to the laboratory seldom contains living leptospiras.

Leptospiras in Cerebrospinal Fluid

As early as 1916, Costa and Troisier[21] described a case of Weil's disease in which the principal symptom was meningitis. Since that time the involvement of the meninges as a constant and sometimes predominant symptom of leptospiral infection has become well known. There is, however, no correlation between the seriousness of the clinical symptoms and the pathologic changes or the presence of leptospiras in the spinal fluid.

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[16] Stavitsky: J. Bact. **50**: 118, 1945.
[17] Randall: J. Am. Vet. M. A. **112**: 136, 1948.
[18] Compt. rend. Soc. de biol. **101**: 486, 1929.
[19] Proc. 4th Internat. Cong. on Trop. Med. & Mal., 1948.
[20] Acta brev. Neeland. 8: 1, 1933.
[21] Bull. et Mém. Soc. Méd. de Hôp., 1916.

In all cases of Weil's disease or canicola fever in Amsterdam, Minkenhof could demonstrate a serous meningitis in the second week, while in only a few cases was the number of cells high. Gsell and Rimpau[22] stress that, in the various leptospiral forms of meningitis, during the first days of the illness no inflammatory changes can be observed, despite distinct meningeal symptoms. They always appear in the second and third weeks, however. Only rarely can the leptospiras be isolated from the spinal fluid. Schüffner and Walch-Sorgdrager[23] found them only in one out of twenty-five cases of Weil's disease with meningeal symptoms. Cultures and animal inoculations are indicated when it is necessary to examine spinal fluid.

Leptospiras in Post-Mortem Material

The demonstration of leptospiras at autopsy may be possible by darkfield examination of smears from the liver, kidneys, or adrenals. In tissues, the silver impregnation by the Krajian method will show the leptospiras in these organs. Cultures will be possible only immediately after death. In all other cases, animal passage is necessary. In chronic animal carriers (rats, dogs, mice, but not rabbits or man), the process in the kidneys is sharply localized to limited parts of the cortical substance; leptospiras will be found in layers on the epithelium of the convoluted tubules. Several parts must be examined before a diagnosis of leptospiral infection can be excluded.[24]

Leptospiras in Surface Water

Surface water is often the source of infection with leptospiras (Weil's disease, mud fever), but it is very difficult to demonstrate these microorganisms in the water. Microscopically, they cannot be distinguished from the nonpathogenic *L. biflexa* which is often found in water.

The only reliable method to date is that of van Thiel and co-workers who bring the warmed water (37° C.) into immediate contact with the tissues of susceptible animals either by bathing guinea pigs with an abraded skin in the suspected water or by letting this flow through the subcutaneous tissue of the animal. By this method even very small numbers of pathogenic leptospiras can be isolated from the water.

SEROLOGIC DIAGNOSIS

If no leptospiras have been isolated from the patient, the diagnosis can be made serologically in a number of cases. The antibodies do not usually appear before the seventh to tenth day of the illness. They then increase rapidly and reach the highest titer in the third or fourth week. The reliability of this method depends to a great extent, however, on the number of strains with which the serum is tested.

If there are no co-reactions and the titer has risen to 1:1,000 or more during the course of the illness, the probable cause of the disease may be established in this way.

Strong co-reactions are, however, often observed, and in these cases absorption tests are indicated (see also Ruys, Minkenhof, and Wolff[19]). Sera of patients infected with the incomplete biotype of *L. icterohaemorrhagiae* may show titers against *L. canicola* which are as high as and at certain moments even higher than against the causative strain. This nonspecific titer rises more quickly and disappears sooner than the specific one, so that in convalescence the titer against the causative strain is always the highest. Even a rising titer against a certain strain in itself, however, is no proof that this strain is the etiological one.

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[22] Schweiz. med. Wchnschr. 74: 51, 1944.

[23] Nederl. tijdschr. v. geneesk. 80: 3000, 1936.

[24] Nederl. tijdschr. f. geneesk. 67: 2018, 1923.

The absorption test gives immediate and more reliable information.

A titer below 1:1,000 may indicate that the causative species was not included in the series of strains used for the tests; it may be only a co-reaction. In convalescence the titer drops but the serum may react positively for years after the illness, be it only in low dilutions (1:30 to 1:300).

In Sumatra, Wolff[25] found the reaction negative one year after the disease in most cases. Low titers always indicate further careful examination.

Patients who have been treated with immune serum at the beginning of the disease form antibodies only very slowly, and high titers are not reached at all.[26] The serologic diagnosis can be conclusive only if all sources of error have been considered and excluded.

Antibodies in Urine

In addition to the antibodies in the blood, the urine also may contain specific agglutinins and lysins.[27] Formalinized cultures should be used for nonsterile urines. The concentration of antibodies has no relation to the presence of albumin in the urine. The titer of the urine is lower than that of blood. In a few cases it was only 1:2, but it may rise to 1:256. Coreactions are much rarer with urine than with sera.

Antibodies in Spinal Fluid

In some cases of leptospira infections, the spinal fluid contains antibodies, but seldom in large quantities. Walch-Sorgdrager[4] found that undiluted spinal fluid was never positive in eleven patients with Weil's disease with and without meningeal symptoms. Gsell,[28] however, mentions positive results in low titers from the second week of the illness on, especially in cases with serous meningitis. The titer varies between 1:2 and 1:100 and sometimes it is even higher than 1:100. A titer of 1:10 is considered proof of the infection.

General Remarks

The various methods outlined above make an etiological diagnosis possible either by isolating the causative agent or by serologic examination. A refined serologic technic is often required. The question has often been raised whether it is necessary to go so far in serologic differentiation and whether the diagnosis of a leptospiral disease without further differentiation would not be enough.

A typical case of Weil's disease may be recognized as such by its sudden onset, high fever, conjunctival injection, muscular pains, jaundice, albuminuria, and meningeal symptoms. The death rate is high. However, the mistake of Noguchi's co-workers, who mistook cases of Weil's disease for yellow fever, is too well known.

Since more attention has been paid to the frequent occurrence of less typical cases, we know that practically all leptospiral infections produce in principle the same clinical picture.

In infections with some strains, jaundice is frequent (Weil's disease, *L. bovis*); with other ones it is rare (*L. canicola, L. grippotyphosa*); other groups are intermediate. Serous meningitis is a predominant feature in some (*L. canicola, L. pomona*); in others it is not often described. The renal and extrarenal disturbances which influence the production and composition of the urine[29] may be severe (*L. icterohaemorrhagiae, L. bovis, L. icterohaemoglobinuriae*) or practically absent (*L. canicola, L. grippotyphosa*). On the whole the differences are, in degree, never essential. The pathogenicity of the various types or species for experimental animals is not the same, but this quality is not essential either. Thus far the only reason, but a very important one, why differentiation seems indicated is the difference in epidemiology. The serologically different strains often show a quite distinct parasite-host relationship and hence a typical epidemiologic behavior.

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[25] Geneesk, tijdschr. v. Nederl. Indië, p. 499, 1936.

[26] Schüffner: Nederl. tijdschr. v. geneesk. **86**: 163, 1942.

[27] van der Hoeden: Ann. inst. Pasteur **56**: 206, 1936.

[28] Bull. Acad. de méd., p. 688, 1946.

L. icterohaemorrhagiae lives in the kidneys of the rat (*Rattus norvegicus norvegicus, Rattus rattus alexandrinus*) where it may persist for the whole of the lifetime of the animal. *L. grippotyphosa* is found in the kidneys of the vole, but after a longer or shorter period the animal may be free of it.[30] *L. canicola* is found only in the dog as a carrier; *L. pomona* in Switzerland is found in infections of swine.[28] *L. hebdomadis* is confined to *Microtus montebelloi* in Japan and to dogs in Medan.

Sometimes it is difficult to decide whether finding a certain animal as a leptospira carrier is mere chance or whether it means that this is the real animal host and the reservoir which enables the parasite to persist. During the last years several diseases have been recognized as leptospiral infections; for example, swineherd's disease in Switzerland (*L. pomona*), jaundice in Palestine (*L. bovis*), a part of the cases with serous meningitis (*L. canicola, L. pomona, L. sejrø, L. grippotyphosa*), and many diseases in the Belgian Congo and Australia. Several animals have been found to be carriers. It will take some time and much work before we know how far minor differences in serology are accompanied by distinct epidemiologic behavior.

The complete and incomplete biotypes seem to behave practically alike in all respects and therefore all groups are considered to belong to the same species.

As long as the epidemiology of the various types or species has not been sufficiently clarified, we think it necessary to keep them separated, even though only provisionally for some.

COLLECTION AND EXAMINATION OF SPECIMENS

It is a mistake for laboratories to make a bacteriological examination for only one organism or for only one group of microorganisms. This procedure should be followed only when making special surveys; for example, when examining for carriers of meningococci or of diphtheria bacilli. It is contrary to the spirit of scientific bacteriology to examine a throat swab for streptococci only when the request or order for examination fails to specify anything else, and thus to overlook the possibility that the patient may be infected with *C. diphtheriae*. Many infections which could be discovered by more thorough examinations of the cerebrospinal fluids, stools, urine, pus, and wounds go unnoticed because the physician, burdened with his clinical and bedside tasks, may neglect to call the attention of the laboratory to the need for carrying out additional procedures.

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[29] Klarenbeek and Schüffner: Nederl. tijdschr. v. geneesk. 77: 4271, 1933.

[30] Schüffner and Bohlander: Ztschr. f. Immunitätsforsch. u. exper. Therap. 104: 237, 1943.

For these reasons, routine methods are recommended in this chapter. These are suggested for every specimen, regardless of special requests. It is self-evident that special procedures must be carried out whenever ordered, in addition to certain routine methods. If this procedure is followed over some length of time, it will bring its reward for the loss of time and material spent in the examination of so-called "negative" specimens.

Whenever possible, specimens must be collected in a sterile manner. This is imperative for blood cultures, cultures of cerebrospinal fluid, pus, material from serous cavities, glands, and urine, not only to prevent contamination, but also, with the exception of urine, for the benefit of the patient. The material must often be collected by the physician. In such cases sufficient sterile collecting utensils must be put at his disposal and early delivery of the specimen to the laboratory must be assured.

BLOOD

Blood Cultures from Infections

Precautions.—

1. Too much blood will inhibit the growth of pathogenic organisms.
2. Too little blood will not contain enough bacteria to obtain a good growth.
3. The culture medium used must be highly buffered.
4. The container in which the medium is placed must have a broad base to insure a good growth.
5. There must be enough culture medium in the container to provide a growth.
6. Care must be taken to eliminate skin organisms from the culture.
7. Care must be taken to eliminate air organisms from the culture.

Method No. 1

Equipment Needed.—

1. Tourniquet.
2. Sterile sponges.
3. Tincture of iodine.
4. Alcohol.
5. Adhesive tape.
6. Alcohol burner.
7. Sterile tube or bottle containing 1 c.c. of 2.5% sodium citrate solution.
8. Bottle containing 100 c.c. sterile broth, M-4 or M-3.
9. One 10 c.c. Luer syringe with a 21 gauge, 1 inch needle, previously sterilized in the dry heat sterilizer at 170° C. for one and one-half hours.

Technic of Making the Culture.—

Place the tourniquet around the arm of the patient above the bend in the elbow.

Locate the vein by palpation.

Disinfect the skin with iodine, in a circular manner, covering a large area of the skin, but taking care not to touch the same area twice.

Prepare the syringe; take care not to touch the needle with the hands. This is accomplished by holding the needle in the paper in which it is wrapped, and placing it on the syringe without touching it.

Flame the tops of the bottles containing the sodium citrate solution and the broth.

Insert the needle into the patient's arm vein, and withdraw 10 c.c. blood.

Remove the tourniquet, then the needle (attached to the syringe) from the vein, holding a sponge saturated with 95% alcohol on the patient's vein.

Immediately eject, with sterile precautions, 5 c.c. of the blood into the broth, the other 5 c.c. into the sterile 2.5% sodium citrate solution. To keep air contaminants from entering the bottles, be sure to flame the tops and hold the bottles in a slanting position while ejecting the blood. Rotate both bottles gently to prevent coagulation of the blood.

Place adhesive tape over the puncture in the patient's arm.

Record the specimen on the laboratory chart.

Place the bottle containing the broth with blood in the incubator.

Melt two tubes of blood agar base M-27 and two of the same type tubes of dextrose agar (M-19). Keep the agar in a water-bath between 44 and 46° C. Agar at this temperature will remain liquid, but will not be hot enough to kill the bacteria which may be present in the patient's blood. If the agar is too hot, it will coagulate the blood which is added to it, giving a ''chocolate'' agar instead of undissolved or uncoagulated blood.

Using all sterile precautions, transfer 1 c.c. of the citrated blood to a tube of blood agar base (M-27), and the same quantity to a tube of dextrose agar (M-19), and rotate both immediately. To prevent contamination with air bacteria, flame the mouth of the tube, flame the pipette (not hot enough to coagulate the blood), hold the tube in a slanting position, and allow the blood to flow into the agar. Blood is mixed with agar in the manner described on pages 1430, 1431. Pour immediately into sterile Petri dishes and allow to harden.

Carry out the same procedure, using 2 c.c. quantities of blood in each tube of blood agar base and dextrose agar, and pour Petri dishes of the mixture. Incubate all four of the Petri dishes at 37° C.

Transfer 1 c.c. of blood also to Brewer's thioglycollate medium M-47 and incubate in the usual manner.

Examination of the Culture.—

Examine both the broth culture and the Petri dish cultures every day for evidence of growth of bacteria. It is not impossible, when the number of bacteria in the blood is low, to find a cloudy broth with no evidence of growth on the Petri dishes. Procedure for further investigation and reporting of apparently negative cultures is given below.

Make a hanging drop of the top fluid of the broth culture. At the same time, remove part of the material from the bottom of this culture with a long sterile capillary (page 1467). Make a Gram stain and observe the morphology and staining characteristics of the organisms.

If bacteria are present in the broth and not on the Petri dishes, transplant some of the broth culture to plates of blood agar (M-28 or M-29) and incubate at 37° C.

If smears or hanging drops show gram-positive cocci, transplant to blood agar plates. Use material both from the top of the broth culture and from the point of contact between the broth and the blood, which has settled to the bottom of the blood culture. Draw out a long capillary from sterile tubing (page 1394). Pick up a portion of the medium from the top of the bottle, from the interface between blood and broth, and from the blood at the bottom of the culture bottle.

Since some streptococci are anaerobic, it is well to transfer some of the culture to Brewer's thioglycollate medium M-49.

Place this material on the surface of a Petri dish of blood agar, and streak in such manner that individual, discrete colonies will develop. Or, add the material to a tube of melted dextrose agar at 45° C., add 1 c.c. of sterile rabbit blood to the agar, rotate quickly between the palms of the hands, and pour with sterile precautions into a sterile Petri dish. Be sure to autoclave the emptied tube, for it may contain virulent bacteria. Using this second method of transplantation, deep as well as surface colonies may be obtained. This enables one better to determine whether or not hemolysis occurs. As soon as the agar has solidified (if the second method is used), or as soon as the plate has been inoculated, incubate at 37° C.

If no subsequent growth occurs, repeat the transplantation. It may be necessary to use a richer culture medium for transplantation of the organisms. (See Method No. 2.)

If the original broth culture contained streptococci, keep it in the refrigerator (not the incubator) until the final examination is complete. This aids in preventing further contamination of the culture from too much handling.

Identify the streptococci if found. If the colonies on blood agar are surrounded by a zone of hemolysis, the organisms are *Streptococcus hemolyticus* group. If the colonies are surrounded by a zone of methemoglobin, showing a faint greenish cast, they are either *Streptococcus viridans* group or pneumococci. For further details on differentiation of types of streptococci, see pages 1487 ff.

Examination for bacteria other than streptococci is given in the alternate method immediately following this method.

Method No. 2

Alternate Method

1. Prepare for operation and draw blood as above.

2. Have on hand large test tubes containing (a) brain heart infusion with agar, p-amino benzoic acid and penicillinase M-4; (b) thioglycollate medium M-47; (c) trypticase soy broth M-3 *or* Huddleson's broth M-24. Chopped meat medium M-12 may be substituted for brain broth infusion.

3. Inoculate tubes with blood directly from the vein *or* collect blood in a sterile container containing 2 c.c. of 2.5% sodium citrate solution, and also inoculate tubes from the citrated blood.

4. Be sure that enough blood is withdrawn to inoculate: (a) *either* 2 tubes of each of the above media (containing 25 c.c. to 30 c.c. of medium per tube) with 2 to 3 c.c. of blood per tube; (b) *or* to inoculate one bottle of each of the above media (containing 100 c.c. of the medium per bottle) with 5 c.c. of blood per bottle.

Precautions.—The media in the tubes or bottles must reach a sufficient depth to allow microaerophilic or anaerobic conditions at the bottom of these containers. If the upper layer of the thioglycollate medium shows a bluish discoloration, heat it for a few minutes at 80° C. Do this, also, with brain heart infusion before adding penicillinase. Media must be cooled to between 20° and 38° C. before inoculating them.

It is not necessary to add p-amino benzoic acid to the thioglycollate broth. Trypticase soy broth and Huddleson's medium may be supplemented with both p-amino benzoic acid and penicillinase.

5. Incubate all tubes or flasks at 37° C. Trypticase soy broth or Huddleson's medium must be incubated in CO_2 atmosphere (pages 1472, 1575).

6. Observe all tubes for three weeks. Make readings and subcultures on the second, third, seventh, fourteenth, and twenty-first day of incubation.

7. Using a sterile capillary pipette, and observing sterile precautions, take material from the interface of blood and broth. If this cannot be done, shake the cultures and use a loopful of the material.

8. Make Gram stains. Proceed according to the findings:

(a) **If gram-positive cocci are seen,** plate on blood agar M-28, M-29, making streaked and poured plates.

(b) **If gram-negative cocci are present,** proceed as under (a) but also streak chocolate blood agar M-31, M-32, M-33, and incubate in CO_2 atmosphere and under normal conditions.

(c) **If gram-negative rods are present,** proceed as under (a) but also streak a MacConkey plate M-63 *or* an eosin-methylene blue plate (M-71 or M-72). Pour tryptose agar plates M-16 and incubate in CO_2 atmosphere. Streak azide or alcohol plates (M-21 to M-23).

(d) In tropical countries, **P. pestis may be present.** In such cases streak on 3% salt agar M-15.

If the blood culture is contaminated, repeat the test, obtaining a new blood culture from the patient immediately. Plate out the contaminated culture if permission is not granted to obtain another culture. If the pathogen can be fished from the contaminated culture, twenty-four hours can be saved in the examination.

Examination of Transplants

I. **If gram-positive cocci are present,** but do not grow under aerobic conditions, try anaerobic cultures on blood agar plates or yeast plate medium (M-14).

II. **If gram-positive aerobic cocci are present.**—

(a) **Staphylococci:** Test as described on page 1485. Be cautious if nonhemolytic, non-coagulase-forming staphylococci are found. They usually are contaminants. For rapid differentiation, hemolysis on blood agar M-28 or M-29, the coagulase test R-4, and fermentation of mannitol in broth M-5 or semisolid medium M-88 are satisfactory.

(b) **Streptococci**: Make preliminary differentiation according to growth and hemolysis on blood agar plates. Methods of differentiation are described on pages 1487 ff. For rapid differentiation, prepare β-hemolytic organisms for serologic classification; inoculate for hippurate reaction R-11 and for test of heat-resistance.

Alpha-hemolytic streptococci must be tested for bile solubility (R-10); fermentation of inulin M-5 or M-6; action on aesculin M-87; hippurate reaction R-11; dextran formation from 5% sucrose, page 1489.

(c) Bile soluble, inulin-negative organisms are suspected of being **Pneumococci.** Aesculin-positive organisms may be **Enterococci.** Inulin-positive, hippurate-negative, dextran-positive organisms are **Streptococcus endocarditicus.**

III. **If gram-negative cocci are present.**—

(a) They may be mutants or variants of gram-positive organisms. These grow well on blood agar plates and show typical reactions, if not changed too much.

(b) Meningococci grow well under aerobic conditions, while gonococci grow better in CO_2 atmosphere. Both give positive oxidase reactions R-18. For further differentiation serum broth with fermentable substances M-6, M-7 are used, together with serologic reactions, page 1502.

IV. **Gram-negative rods.**—

(a) **If gram-negative rods**, which grow well under aerobic conditions, are present, colonies from MacConkey or eosin-methylene blue plates are used. Test according to the schedule outlined under stool examination (pages 1632 ff.). If coliform organisms are present, test their IMViC reaction (page 1536).

(b) *Pasteurella tularensis* grows very slowly. It may be present in the blood during the first week of the disease.

(c) *Brucella abortus* grows only in CO_2 atmosphere, on tryptose agar, while other Brucellae grow well on this medium in a normal atmosphere. Differentiation is carried out by growing on liver infusion agar plates with the addition of dyes (M-26a) and by agglutination.

V. **If gram-positive bacilli are present,** aerobic sporeformers are usually indicative of contamination.

(a) Anthrax bacilli are differentiated according to the outline of Stein (page 1547). The most important reactions are pathogenicity for guinea pigs and lack of hemolysis.

(b) Anaerobic gram-positive organisms are classified as sporeformers (Clostridia) and "others." The latter are difficult to distinguish. Proceed according to pages 1554 ff.

VI. **For Leptospira,** see pages 1587 ff.

If a **blood clot** is received, pour it into a sterile 50 c.c. syringe; mount a small needle on the syringe; expel the air through the needle; discard the needle, and press the contents out into a flask containing culture medium.

The alternate method of bacteriologic examination of blood is recommended for routine procedures, if the patient has received sulfa drugs. Culture media containing p-amino benzoic acid are then used. If the patient has received penicillin, use media containing penicillinase (M-20). About 0.03 gm. cysteine hydrochloride or sodium thioglycollate will counteract streptomycin in 100 c.c. of medium.

ANIMAL INOCULATION WITH LAKED BLOOD

Collect 20 c.c. of venous blood and transfer immediately to a flask or bottle containing 90 c.c. of sterile distilled water.

Add a few drops of sterile 5% solution of tannic acid to form a precipitate.

Centrifuge at high speed.

Inject the entire sediment into a guinea pig.

Tag the pig. Record the number of the guinea pig, the date and number of the specimen, and the weight of the guinea pig. Proceed in the usual manner.

CEREBROSPINAL FLUID*

Whenever possible, collect the spinal fluid in three different sterile tubes. Discard the first tube if it contains blood. Use the second tube for cell count and chemical examination; use the third for bacteriologic tests.

Spinal Fluid in Meningococcus Meningitis†

Turbidity.—Meningitic spinal fluid is very turbid.

Globulin Test.—Make both the Nonne-Apelt and the Pandy tests for globulin.

Meningitic spinal fluid gives a strong positive globulin test.

Lange Colloidal Gold Test.—The curve in meningitis is typical. See Lange's colloidal gold reaction, pages 1253, 1255.

Cell Count.—The cell count in bacterial meningitis is unusually high. (See page 1243.)

Routine Procedure

The routine procedure in the bacteriologic examination of spinal fluids consists of two procedures: (1) examination of a Gram-stained slide of the sediment; and (2) proper culture methods.

Centrifuge the spinal fluid and examine the sediment after staining with the Gram stain.

At the same time, inoculate 1 to 2 c.c. portions of the cerebrospinal fluid into (a) one test tube containing brain infusion broth with p-amino benzoic acid and penicillinase M-4, and (b) one tube of thioglycollate medium M-47. Prepare the tubes as for blood cultures.

Certain information as to the proper procedure to follow can be gained from examining the Gram-stained slide.

1. **If gram-negative cocci or rods are found in the smears,** streak on (a) chocolate agar M-33; (b) blood agar prepared with human or rabbit blood, M-28, M-29.

Incubate duplicate plates, one set in CO_2 atmosphere, the other in normal atmosphere. Gonococci grow better in CO_2 atmosphere, and on chocolate agar.

Meningococci may be typed by serologic methods (page 1502) as soon as possible. Fermentation tests (page 1501) are helpful in differentiating them.

H. influenzae grows better on blood agar. Transfer the growth suspected of being *H. influenzae,* to a second plate. Streak over the second plate (cross-streaks) with a culture of *Staphylococcus aureus.* The *H. influenzae* grows better in the vicinity of Staphylococcus colonies (satellite phenomenon).

If large colonies are present on blood agar, transfer them to triple sugar iron agar M-80, and proceed as under "stool examination." Coliforms are differentiated by the aid of their IMViC reaction (page 1536).

One must keep in mind that Klebsiellae (page 1504) may also be found.

2. **If gram-positive organisms are found,** proceed as under "blood cultures."

3. Cultivation of cerebrospinal fluid on the chorio-allantoic membrane of the developing chick is an excellent method for detecting some infectious agents (viruses). See pages 1473 ff.

4. **If infections with yeasts or molds are suspected,** or if such organisms are seen in the stained smears, inoculate:

(a) Three blood agar plates M-28, M-29.

(b) Three plates of Sabouraud dextrose agar M-102.

Seal two of the blood agar plates and two of the Sabouraud plates with Plasticine or adhesive tape. Incubate one of the blood agar plates and one of the Sabouraud plates (sealed) at 37° C., the other plate (sealed) at room temperature.

Incubate the remaining blood agar plate and the Sabouraud plate (not sealed) under anaerobic conditions (pages 1469 ff.).

If only limited facilities are available, media in tubes may be used, provided the cerebrospinal fluid does not contain other microorganisms.

For further explanation, see under "Skin."

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*See Chapter VII, Puncture Fluids, for other tests.

†See pp. 1260 ff. for other tests of meningitic spinal fluid.

5. **Rarely, P. tularensis may be present.** Grow such organisms on cystine agar M-30.

6. **In exceptional cases, Listeria monocytogenes will be found.** Culture the spinal fluid on blood agar M-28, M-29.

7. **Examination for tuberculosis.** Centrifuge the cerebrospinal fluid and streak the sediment directly on at least three media favorable for the growth of *Mycobacterium tuberculosis.* The author prefers Petrik medium (M-58). The medium of Dorset (M-59) or those of Petragnani (M-61) *or* Loewenstein (M-60) and Dubos (M-62) represent a good selection. More recent media are those of Tarshis (M-58^a) and Whalen (M-58^b).

Examine the sediment of the centrifuged specimen after staining with acid-fast staining methods.

If animal inoculation is desired, inject a guinea pig (page 1647).

8. **For Leptospiras,** see pages 1587 ff.

PUS FROM LOCALIZED ABSCESS

Assemble the following materials:

Sterile swab.
Sterile test tube.
Alcohol burner.
Iodine or Merthiolate tincture.
Alcohol.
Sterile sponges
Sterile scalpel.

Disinfect the skin over the abscess and allow to dry.

The attending physician makes the incision into the abscess.

Remove and discard the first pus from the abscess.

Without touching the skin, carefully remove a portion of the pus with the sterile swab. A platinum wire may be used instead of the swab. The wire must be heated and allowed to cool before using. Put the swab into the tube and rush it to the laboratory.

Inoculate (a) one tube of thioglycollate broth M-47; (b) one plate of blood agar M-28, M-29 or M-21 to M-23; (c) one tube of broth M-2.

Make smears and stain with Gram stain. If **staphylococci are present,** proceed with the coagulase test.

1. **If gram-negative rods are present,** streak on the surface of an eosin-methylene-blue plate M-71, M-72. This medium is selected because Protei do not spread on it if the surface is not wet. Proceed with the examination usually made for gram-negative rods as suggested under ''stool examinations.''

2. **If gram-positive bacilli are present,** proceed as for ''Anthrax'' and ''Clostridia'' outlined under *blood.* Nonsporeforming anaerobes are difficult to classify.

3. **If mycotic elements are seen,** inoculate 3 blood agar plates M-28, M-29, and 3 Sabouraud dextrose plates M-102, as outlined under ''spinal fluid.''

4. **If, as very rarely occurs, Past. pestis is present,** examine for this organism as described under *Past. pestis* on page 1568.

5. **If a tuberculous lesion is suspected,** the physician removes pus from the abscess by puncture, and submits it to the laboratory for examination. The pus is then treated as sputum.

A special method of examination of pus for tubercle bacilli is as follows:

In the examination of abscess pus for tubercle bacilli, it must be remembered that these organisms decolorize much more rapidly when present in pus than when found in other specimens or in cultures.

A smear is made and stained with the Ziehl-Neelsen stain and examined for tubercle bacilli. In tubercular pus, many mononuclear elements (monocytes) together with degenerated cell elements are present. There is also much granular material. However, the absolute differentiation of tubercular pus from other abscesses cannot be definitely made from the cellular content.

A portion of the pus may be injected into guinea pigs for evidence of tubercle bacilli.

MATERIAL FROM SEROUS CAVITIES

Material from serous cavities is examined like pus. Attention must be given, however, to its physical, chemical, and cytologic properties (see Chapter VII and pages 2156 and 2184 ff.).

A special method for detection of tubercle bacilli in pleural fluid is as follows:

Unless pleural fluid is centrifuged immediately after aspiration, a coagulum will form.

(1) Before coagulation takes place, add a small amount of calcium chloride to the pleural fluid, or defibrinate the fluid.

Add an equal volume of distilled water, or a small amount of 15% Antiformin.

Centrifuge at high speed.

Make smears. Fix in the flame. Stain with the Ziehl-Neelsen stain and examine for tubercle bacilli.

(2) If the pleural fluid has formed a coagulum, proceed in the following manner:

Separate the clot from the pleural fluid.

Add to the clot equal volumes of N/1 sodium hydroxide and 15% Antiformin solution.

Shake thoroughly.

Digest for thirty minutes.

Centrifuge at high speed for fifteen minutes.

Decant and discard the supernatant fluid.

To the sediment, add a few drops of N/1 hydrochloric acid.

To isolate *Streptobacillus moniliformis,* inoculate fluid medium M-118 with fluid from the joint.

After 2 or 3 days, streak the growth to the solid M-118 medium.

Either make smears, inoculate tubes of Loewenstein medium (M-60) or Petrik's medium (M-58^{a}) or Petragnani (M-61), Tarshis or Whalen (M-58^{a} and ^{b}), or inoculate guinea pigs.

WOUND SECRETIONS AND FISTULA*

Due to the variety of methods used in the treatment of wounds, the material available for examination may be a tissue specimen collected by biopsy, swabs, aspirated fluid, or fragments of skin.

1. Specimens, particularly tissues, should be shipped in the frozen state.

In all other cases it is recommended that the specimen be mailed in screw-capped tubes containing *either* chopped meat medium (M-12) *or* fluid thioglycollate medium (M-48). It is essential that the containers be large enough (e.g., 50 c.c.) and that at least 20 c.c. of the medium be in each of them.

For particular instructions, see Clostridia, page 1554.

2. Upon arrival in the laboratory, chopped meat medium (M-12) is inoculated with the frozen specimens.

All tubes, i.e., those inoculated at the bedside and those inoculated in the laboratory, are incubated for 24 hours at 37° C.

3. After incubation, 1 to 1.5 c.c. of the liquid are withdrawn with a sterile pipette and placed in thioglycollate broth (M-48) in 5 by ⅝ inch tubes, containing about 6 c.c. per tube.

The original liquid is kept at room temperature for reference, while the thioglycollate broth subculture is incubated for 24 hours at 37° C.

4. From tubes *3,* inoculate the following plates:

1 eosin-methylene blue plate (M-71 or M-72)
1 tellurite blood agar plate (M-41 or M-42)
2 yeast blood agar plates (M-14)
1 sodium azide-chloral hydrate plate (M-22)
1 modified Nagler plate (M-57)

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*The method given here is that used in the 406 Medical General Laboratory of the U. S. Army, as described in its *Annual Historical Reports* for 1950, 1951, and 1952. We are indebted to Mr. Th. Wetzler, Research Bacteriologist, and Capt. J. Marshall for this outline.

5. Incubate the eosin-methylene blue, the tellurite, and one of the yeast blood plates aerobically for 24 hours at 37° C.

Incubate the other plates anaerobically for at least 40 hours, but not more than 60 hours, at 37° C., using anaerobic methods of incubation as recommended under Clostridia, page 1555.

6. Perform an acrolein test (R-19) in tube *3*.

7. Keep in mind that many aerobic organisms will grow well under anaerobic conditions. Thus, not all colonies grown on the plates contained in the anaerobic jars are Clostridia.

Gram-negative organisms will not grow on the tellurite and the azide-chloral hydrate plates. They will be picked up best from the eosin-methylene blue plate and examined as under *Stools*, pages 1631 ff.

The hemolytic reactions of cocci cannot be recorded from plates containing carbohydrates. Subcultures on plain blood agar plates (M-28 or M-29) must be examined for this purpose.

Gram-positive aerobic rods will not grow on eosin-methylene blue plates. Cocci, however, may form small colonies with a purple central dot on them.

8. Read the acrolein test (R-19) on the modified Nagler plate.

9. All gram-positive bacilli which show negative or negligible growth on aerobic yeast blood agar plates are tentatively classified as Clostridia and studied further according to the outline for that genus, pages 1548, 1557 ff.

SPUTUM

The examination of sputum consists of two procedures: macroscopic and microscopic examinations.

The macroscopic examination comprises the following examinations: (a) the total volume in twenty-four hours; (b) the color; (c) the odor; (d) the consistency; (e) the presence of nasal mucus, carbon, pus, blood, cheesy particles, food, or vomitus; (f) layer formation; (g) lung stones; (h) bronchial casts; (i) Dittrich's plugs.

A microscopic examination is made on the stained and the unstained specimen. The unstained specimen is examined for (a) elastic fibers; (b) Curschmann's spirals; (c) Charcot-Leyden crystals; (d) myelin globules; (e) pigmented cells; (f) ray-fungus (*Actinomyces bovis;* (g) pus cells; (h) epithelial cells; (i) blood. The stained specimen is examined for (a) pus cells; (b) epithelial cells; (c) tubercle bacilli, reported according to the Gaffky scale (see page 1609); (d) other pathogenic bacteria, such as pneumococci, Klebsiellae, staphylococci, streptococci, etc. Other bacteria which may contaminate the specimen are not usually reported, unless it is necessary to convey to the physician the fact that the specimen is badly contaminated either with air organisms, or with saliva. In such cases, report sarcinae, hay bacilli, yeast cells, molds, higher bacteria, spirochetes, fusiform bacilli. It is very important to examine the sputum for (e) pathogenic yeasts and molds, and (f) for eggs of animal parasites.

Collecting the Specimen

A one- or two-ounce sterile wide-mouthed bottle is used. This has been thoroughly washed, and sterilized in the dry heat sterilizer at 170° C. for two hours. The first specimen of the morning is collected. The patient is instructed to rinse his mouth well with water, then to cough up a specimen from his lungs, not to expectorate saliva from the mouth. Discard the first few specimens, thus avoiding food remnants.

Some laboratories prefer 24-hour specimens for *M. tuberculosis* examination.

Macroscopic Examination

1. **Total Volume in Twenty-Four Hours.**—Normally the quantity of sputum is negligible. It is very important in some cases, particularly in checking the progress of a tubercular patient, to measure the volume of the twenty-four-hour specimen of sputum.

2. **Color.**—Pour the specimen into a Petri dish on a black surface. The color may be gray, yellow, green, red, brown, or black. Pure mucoid expectoration is transparent and colorless. Leukocytes impart an opaque characteristic to the sputum, the specimen becoming green upon standing, if pus is present. In infections with *Pseudomonas aeruginosa (B. pyocyaneus)*, in jaundice, and in pneumonia during lysis and subacute pneumonia, the specimen may be green. A green sputum is also noted at times in pulmonary carcinoma, in bronchiectasis, and in pulmonary abscesses. A red color in the sputum may, and usually does, indicate the presence of blood. Bright red blood has recently been expressed, while the brown colors come from blood which has been retained in the lung. Bright red streaked sputums usually are seen in pulmonary tuberculosis, although this is not a diagnostic point. In pulmonary gangrene the sputum is a dirty brownish color, due to methemoglobin and hematin. In chronic passive congestion due to disease of the heart the sputum is also brown. The typical sputum of pneumonia is a rusty or chocolate color, often referred to as ''prune juice'' sputum. Vomited blood that has been swallowed is black and acid.

3. **Odor.**—Fresh sputum has no odor normally. In pulmonary gangrene and putrid bronchitis, a foul odor due to decomposition by anaerobic bacteria is noted. Old sputums undergo putrefaction rapidly.

4. **Consistency.**—Tenacious sputums usually contain mucus. Thick purulent masses that sink in water are found in sputum from cases of chronic tuberculosis. Sputums consisting of pure pus, which may be quite fluid, are found in cases of acute pulmonary gangrene, pulmonary abscesses, putrid bronchitis, and following perforations into the lung in empyema.

5. **Nasal Mucus, etc.**—Examine the sputum in the Petri dish. Cheesy masses may sink to the bottom, and may vary in size from a millet seed to the size of a pea. In far-advanced stages of pulmonary tuberculosis, and in acutely progressive tuberculosis, these particles are found. They usually contain numerous tubercle bacilli. Do not confuse them with food particles, or Dittrich's plugs (9, below).

6. **Layer Formation.**—Observe whether the sputum separates into three sharply defined layers when a large volume is standing in a tall vessel. This characteristic of sputum is seen in cases of bronchiectasis, gangrene, and abscess of the lung.

7. **Lung Stones.**—Pour the specimen through a fine wire gauze screen. Small calcified nodules of tuberculous tissue may be found. They are white or gray.

8. **Bronchial Casts.**—Transfer suspicious particles to water, float them, then mount on a slide with a cover glass, and examine with the low power

magnification. They are recognized by their imperfect branching structure. They are found in tuberculous hemoptyses, and are also demonstrated in croupous pneumonia immediately following resolution. They are white when caused by true fibrinous bronchitis, but not uncommonly are yellowish brown or reddish yellow. Most of the branches appear to be cylindrical, but some of them are flat.

9. **Dittrich's Plugs.**—They are yellowish or gray caseous masses usually about the size of a pinhead, but sometimes as large as a bean. They emit a foul odor when crushed. Do not confuse them with cheesy particles. They consist microscopically of granular débris, globules of fat, fatty acid crystals, and bacteria. They are seen sometimes normally, but more frequently in cases of putrid bronchitis and bronchiectasis.

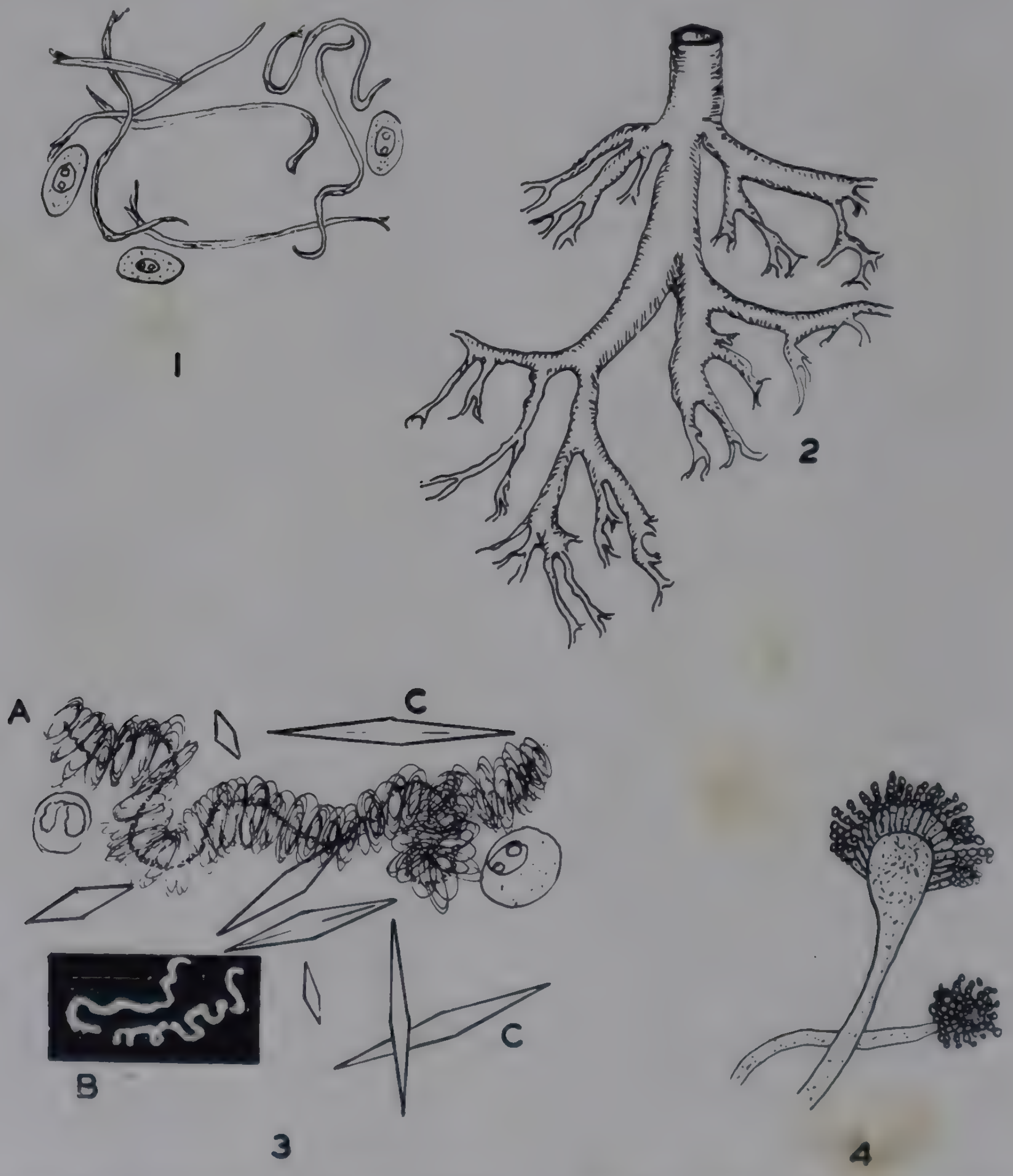

Fig. 389.—Unstained sputum: 1, elastic fibers; 2, bronchial cast; 3, A, Curschmann's spirals magnified and B, not magnified, C, Charcot-Leyden crystals; 4, aspergillus.

Microscopic Examination

(A) Unstained

A cover glass preparation is made by placing a drop of sputum on a glass slide and covering it with a thin cover glass. Examination is made with the low power magnification first, then with the high dry lens. Look for the following:

1. **Elastic Fibers.**—Bits of elastic tissue may be coughed up in the course of acute destruction of the lung parenchyma, as in cavity formation. Select small bits of necrotic

tissue, colored particles, or purulent masses. Place them on a glass slide, and tease with a drop of potassium hydroxide solution to dissolve the caseous matter. Press a cover glass over this preparation, and examine microscopically. The smears must be thin. They are coils of highly refractile, delicate, smooth, elastic fibers, with frayed ends. Do not confuse them with food particles and cotton fibers. Food particles are coarse, and have sharply cut angular or rounded ends, while elastic fibers are flat and twisted.

2. **Curschmann's Spirals** are found in true bronchial asthma. They consist of a twisted network of extremely delicate fibers containing epithelial cells and a number of leukocytes. Select a whitish, threadlike formation, place on a slide, cover with a cover glass, and examine with the 16 mm. objective. Typical spirals will be seen. They can sometimes be seen with the naked eye.

3. **Charcot-Leyden Crystals** often appear in the sputum after it has been coughed up and has stood for some time, even though they are absent immediately after collection of the specimen. They are pointed at the ends, and are colorless, often needle-like. On cross-section, they are hexagonal. They are present in bronchial asthma.

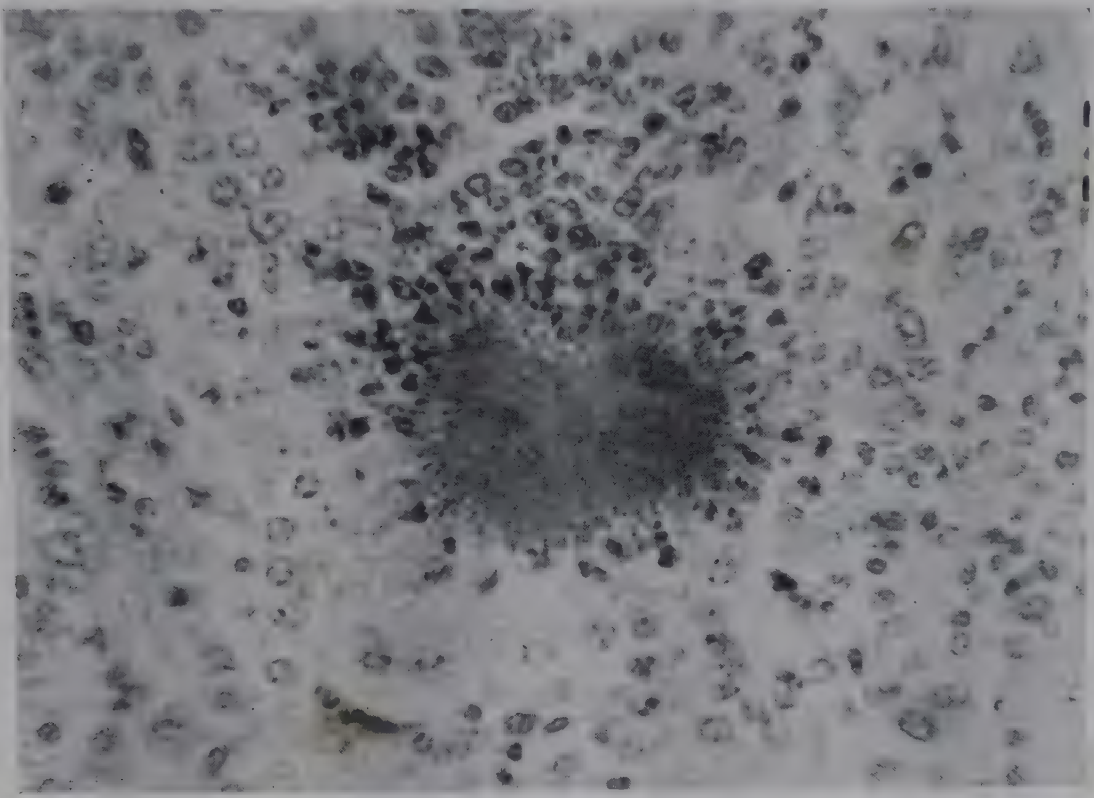

Fig. 390.—*Actinomyces bovis* (×1500). (Courtesy Dr. Oscar Felsenfeld.)

4. **Myelin Globules** are colorless, round, oval, or pear-shaped globules of various sizes, resembling fat droplets, often showing peculiar concentric or irregularly spiral markings. They have no clinical significance.

5. **Pigmented Cells.**—Heart-failure cells contain rounded clumps of brown-pigmented granules. The granules are present in large mononuclear cells.

6. **Ray-Fungus.**—Search the sputum carefully for small gray or yellowish "sulphur" granules. Crush one of these granules between a slide and a cover glass, and examine with the 16 mm. objective. The fungus is composed of a network of threads in a radial arrangement. Fig. 390. (See Actinomycosis, pages 1776 ff.)

7. **Pus Cells** and other leukocytes are characteristic.

8. **Epithelial Cells.**—The majority of epithelial cells in the sputum are the large squamous type. (See page 96.) They are easily recognizable. They are insignificant unless appearing in great numbers.

9. **Blood.**—Erythrocytes are characteristic when present. They are biconcave disks. They may be significant if the source of bleeding is within the lung. The macroscopic

examination of sputum for blood is more important than the microscopic. In tuberculosis, bloody expectoration is very important. Hemoptyses are frequent in bronchiectasis, lung abscess, cancer, silicosis, and heart disease.

Bleeding often arises from sources outside the lung. Streaks mingled with mucus and saliva may originate in the gums, pharynx, or tonsils. In severe bronchitis with tenacious exudate, the sputum is often streaked with blood.

(B) Stained

Pour the specimen into a Petri dish.

(a) Direct Smear

Search the specimen in the Petri dish for ''cheesy'' particles if examining for tubercle bacilli.

Spread the particles in an even layer over the surface of a glass slide.

Allow to dry in the air. Fix in the flame. Stain either with the cold Ziehl-Neelsen stain (24 hours), or with the hot Ziehl-Neelsen stain (3 minutes). (Page 1411.)

Stain other slides with Gram stain.

Examine with the oil immersion objective for the following:

1. **Pus Cells.**—Pus cells appear characteristically when stained. (See unstained examination above.) They stain blue.

2. **Epithelial Cells.**—Epithelial cells stain blue with the Ziehl-Neelsen stain, and are characteristic.

3. The relative **Number of Bacteria** is judged by the examiner. Number of bacteria is recorded as very occasional, occasional, few, moderate number, many or very many.

4. **Contaminants.**—Nonpathogenic yeasts and molds, hay bacilli, salivary organisms, diphtheroids, sarcinae, and other common contaminating organisms of sputum are not reported unless the sputum is so badly contaminated as to render an accurate examination impossible.

5. The **Lung Fluke** or *Paragonimus westermani* is sometimes found in sputum, since it inhabits the lung producing small cavities. The *Paragonimus westermani* (see Parasitology chapter) produces a moderate degree of hemorrhage at times. The eggs of this worm may be found in the sputum and also in the stool from swallowed sputum. The worms are seldom found except at autopsy in the course of the examination of the lungs. They are oval, red-brown, with a flat ventral surface and measure 8 to 10 by 4 mm. The eggs are very thin-walled, smaller than the Diphyllobothrium eggs and at one end have a small operculum.

6. If **Pneumococci** are present and typing is desired, proceed with the typing. When the number of organisms is small, inoculate a mouse and use the peritoneal exudate for typing (page 1494).

7. **Klebsiella pneumoniae** is cultured (a) on eosin-methylene blue agar M-71, M-72, on which it forms large, moist colonies; (b) or on blood agar plates.

8. Examination for **P. tularensis** and **Anthrax** is described in the section under blood cultures.

9. If examining for **Pasteurella pestis**, isolate on yeast agar M-14.

10. To examine for **molds** and **yeasts**, use the plating method, described under ''Fistula.'' Great care must be exerted when evaluating these cultures. For this type of examination, instruct the patient to rinse his mouth with a weak solution of alcohol in water before collecting the sputum specimen.

11. **Examination for Tubercle Bacilli.**—

The sputum is collected in the morning, in waxed paper cups. Some prefer sputum collected for a 24-hour period.

If the ''sputum'' delivered to the laboratory consists only of oral saliva, note this fact on the report.

Select a particle of the sputum and streak it over a slide which has never been used before, carefully rubbing it in the slide, so that a uniform smear results. Repeat this process with another particle collected from a different part of the sputum. Be careful not to scratch the slide with the loop. Carbol fuchsin often accumulates in these scratches and may resemble acid-fast organisms. New slides are used for the same reason.

Examine under the oil immersion objective and report the tubercle bacilli according to the Gaffky scale below. Count all the tubercle bacilli in 10 to 20 consecutive fields and take the average per field.

Gaffky Scale for Reporting Tubercle Bacilli in Sputum

Gaffky I —Only 1 to 4 bacilli in the whole preparation.
Gaffky II —Only 1 bacillus on the average in many fields.
Gaffky III —Only 1 bacillus on the average in each field.
Gaffky IV —2 to 3 bacilli on the average in each field.
Gaffky V —4 to 6 bacilli on the average in each field.
Gaffky VI —7 to 12 bacilli on the average in each field.
Gaffky VII —13 to 25 bacilli on the average in each field.
Gaffky VIII —About 50 bacilli on the average in each field.
Gaffky IX —About 100 bacilli on the average in each field.
Gaffky X —Innumerable bacilli on the average in each field.

Fluorescence microscopy (below) is also used to identify tubercle bacilli.

If the microscopic examination does not reveal tubercle bacilli, proceed with digestion and concentration of the specimen.

(b) Concentrated Smear

Concentrate the specimen (page 1613).

With the sediment make two smears. Stain with Ziehl-Neelsen stain.

Examine for tubercle bacilli.

This examination is carried out only when tubercle bacilli have not been found in the direct smear.

(c) Examination for Leukocytes

(a) **Eosinophiles.**
Eosinophiles may be detected in the smear by staining with the Giemsa, Wright, or Hansel stain. They are characteristic. (See pages 640 and 1353.)

(b) **Monocytes.**
Monocytes may be recognized by their typical form in the routine Ziehl-Neelsen stain, or they may be stained with either the Giemsa or other blood stain. The Giemsa stain is preferable, since it stains monocytes characteristically. (See page 641.)

Fluorescent Microscopic Demonstration of Tubercle Bacilli

Richards, Kline, and Leach[1] described a new and useful method of increasing the number of positive demonstrations of tubercle bacilli in sputum by means of fluorescent microscopy. Fluorescence is a property of certain substances whereby the absorption of invisible, ultraviolet radiation stimulates the emission of visible light. Exactly how this takes place is not clearly known, but it presumably involves electronic rearrangement. Since acid-fast organisms will absorb and retain certain fluorescent acid-fast materials in the same manner that they retain acid-fast dyes, the principle affords a new approach to the problem of demonstrating acid-fast organisms. In practice it seems to have advantages over other methods.

The spontaneous fluorescence of some substances was observed by Köhler in 1904 while experimenting with the ultraviolet dark-field microscope. Kaiserling demonstrated the whitish-blue fluorescence of a suspension of human tubercle bacilli in 1917. A bovine strain was greenish. A number of other reports have since been made, followed by Kline and Leach, the present writers, who reported in 1940 the fluorescence technic suitable for demonstrating tubercle bacilli, lepra bacilli, and nonpathogenic acid-fast organisms.

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[1] Am. Rev. Tuberc., Sept., 1941.

Apparatus.—

For this demonstration a simple fluorescence apparatus for use with monocularmicroscopes has been designed by Richards. It consists essentially of light from a source rich in ultraviolet rays, passed through a blue ultraviolet transmitting filter, reflected through the microscope from an aluminum-surfaced mirror, and finally passed through a yellow contrast filter placed in the eyepiece. The high amperage, low voltage, concentrated filament bulb is satisfactory for this purpose. Filters are chosen so that the ultraviolet radiation and blue light transmitted by the lamp filter are absorbed by the yellow eyepiece filter and the field appears dark. Bacteria retaining auramine after staining appear as self-luminous, golden-yellow bodies with a distinctive morphology. They appear somewhat like dark-field preparations. The bacteria are easily recognized at comparatively low magnifications and can be found on a slide with a 16 mm. objective and the 20× compensating eyepiece. They can be seen in sufficient detail to be recognizable with the 8 mm. objective and the 20× compensating eyepiece or with a 4 mm. objective and a 10× eyepiece. The permanent glass filters are of American manufacture. The blue filter transmits ultraviolet, violet, and a small amount of blue rays. The yellow filter emitted by the auramine-stained tubercle bacilli is approximately of wave lengths 520 to 650 mμ. This method is not adapted to all types of fluorescence microscopy but serves for tubercle bacilli. The yellow filter used in the eyepiece prevents ultraviolet radiation from interfering with the visual picture and protects the observer's eyes from damage which might result from ultraviolet radiation.

Technic.—

Heat-fixed films are stained in the cold for three minutes with the following solution:

Auramine-O, made as follows: auramine-O, 1 gram; phenol absolute, 3 c.c.; and freshly distilled water, 97 c.c. This solution should be kept in the dark and made fresh every two weeks. It forms a slight precipitate without apparent loss of staining property.

Rinse the film in running water and decolorize for two minutes, and again for five minutes, with two applications of the following solution: concentrated hydrochloric acid, 4 c.c.; sodium chloride, 4 grams; 70 per cent alcohol, 1 liter. Decolorization may be continued much longer if desired, even for twenty-four hours. The slide is rinsed in running water, dried, and examined with the 4 mm. objective and a 10× ocular, or with an 8 mm. objective and a 20× ocular.

In films made from concentrates of sputum by sodium hydroxide digestion, practically no fluorescent material is seen, except the auramine-stained bacilli, so that the stain looks very much like a pure culture. Direct films from sputum or spinal fluid contain many fluorescent inflammatory cells which hinder the examination. In such cases, Hermann* suggests counterstaining for a few seconds with 0.1 per cent potassium permanganate solution followed by Loeffler's methylene blue, to make the cells so dark a blue that they become invisible.

Hughes Modification of Fluorescence Microscopy

An improved stain for fluorescence microscopy was suggested by Hughes.[1] He called renewed attention to the fact that the main factors preventing the wider use of this method has been the difficulty of adapting it to the use of the binocular microscope, as the illumination usually available is sufficient only for a monocular instrument. Since the proper style of lamp using a more powerful source of ultraviolet light is not as yet available, he tried to overcome this by improving the standard carbol-auramine stain. The "triple" stain finally developed has been found to give very satisfactory results, the brilliance of the fluorescing bacilli being increased and their contrast with the background emphasized because of a selective color effect.

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*Hermann, W.: Deutsche med. Wchnschr. 12: 329, 1939.

[1] Hughes, G. C.: Tubercle 27: 91, 1946.

Reagents.—

Hughes Auramine Stain.—

Auramine O S	0.4	gm
Rhodamine B S	0.2	gm.
Acridine yellow, c.p.*	0.1	gm.
Liquid phenol (b.p.)	4	c.c.
Glycerine	30	c.c.
Distilled water	66	c.c.

Place the liquids in an Erlenmeyer flask, and add the dyes. Shake, heat to boiling point, and allow to cool.

Acid Alcohol.—

Add 0.5 c.c. of concentrated hydrochloric acid to 100 c.c. of 70 per cent ethyl alcohol.

Technic.—

Prepare very thin uniform smears, or the organisms will not stain evenly. No disinfectant should be present. Do not overheat in fixing.

Warm the stain to 70° C. by immersing the dropper bottle in a bath of hot water or by heating over a small flame.

Pour on the stain and leave for five minutes.

Wash off with tap water.

Decolorize with acid alcohol for five minutes.

Wash with tap water and allow to dry.

The tubercle bacilli are seen as brightly shining orange-red rods on the pale yellow-green background formed by the other constituents of the smear. Visibility is more marked, likewise the contrast, than with the orthodox auramine stain. No counterstains are necessary as they always cause dulling of the bright fluorescence of the organisms. This also makes examination of negative smears less tedious. Even a few bacilli are easily detected by this stain.

Where numbers of smears are to be examined in a laboratory, this method of examination is invaluable in saving time, eliminating eyestrain, and promoting accuracy. It is not recommended in a laboratory having only an occasional smear to examine.

Simplification of Fluorescence Microscopy

Graham[2] has very properly called attention to the fact that most of the literature on this subject makes it appear that this is a very difficult and complicated technic. He states that the insistence that a polished front-surface aluminum mirror must be used in place of the usual microscope mirror, in the belief that a silver surface absorbs the ultraviolet, is unfounded.

The simplified technic of Graham recommends a powerful, concentrated source of light, like a carbon arc or a 500-watt projection bulb, fitted with a condensing lens of about 7 cm. focal length with the blue filter in front of the condenser. This supplies an illumination bright enough to permit examination of films in a room darkened only by drawing the window shades. A satisfactory bulb for the purpose has been found in the 500-watt G.E. projection bulb operating on 110 to 115 volts. This is mounted in a ventilated sheet metal lamp box improvised in the laboratory. A condenser may be obtained from an old arc light, but a pair of ten-cent store reading lenses in tandem has proved equally useful. The proper relation of the lamp to the condensing lens is determined by trial and error, checking the effect of changes by the appearance of the field in the microscope. It has been found that the lamp should be set with the plane of the filament coils at an angle of about 30° to the optical axis so that the images of the coils will nearly overlap, as seen in the lighted field of the microscope. The housing should be large and provided with ample air inlet and outlet to dissipate the heat rapidly. It is better to place the filter in front of the condensing lens rather than behind it. The beam of light should practically cover the mirror of the microscope, and its location is best seen when the mirror is half covered with a card during adjustment.

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*The acridine yellow (2:8 diamino-3:dimethyl acridine) used was prepared by Dr. Adrian Albert, Department of Organic Chemistry, University of Sydney. Commercial samples have not always proved satisfactory. The auramine and rhodamine B were ordinary commercial type dyes obtained from Imperial Chemical Industries, Ltd.

[2] J. Lab. & Clin. Med. 27: 1188, 1942.

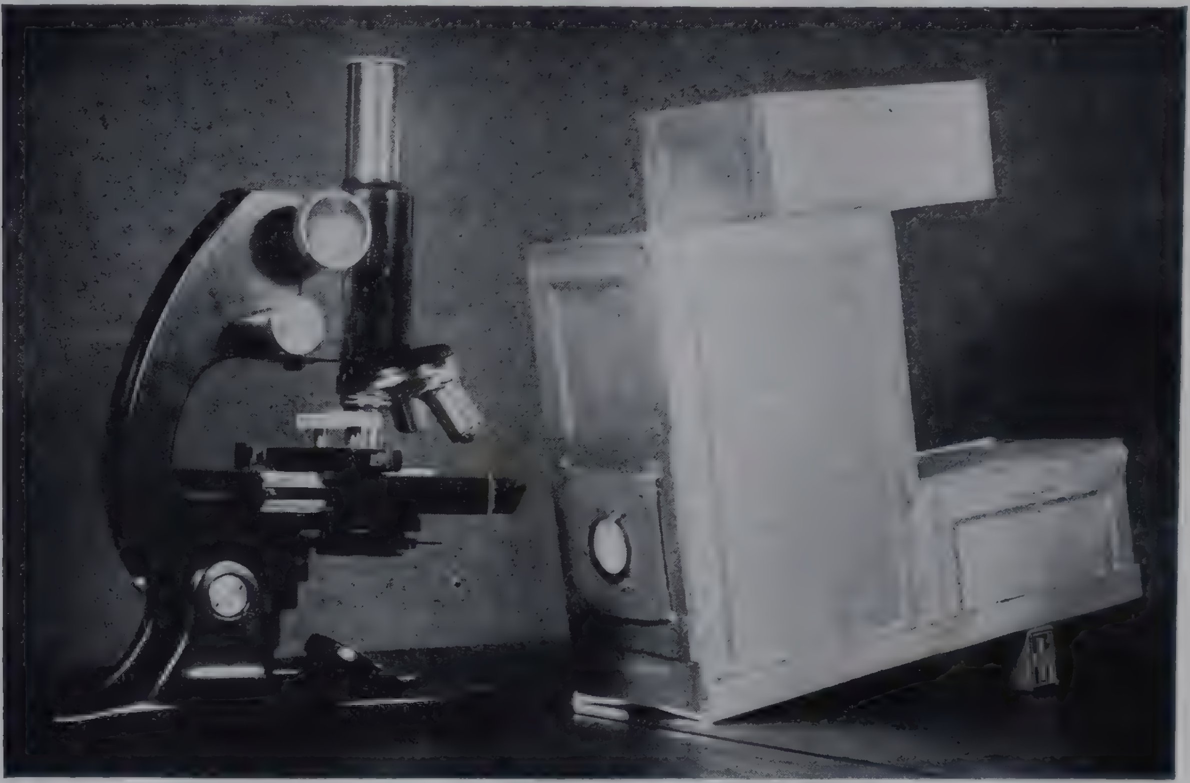

Fig. 391.—Graham's improvised light housing for fluorescent microscopy.[2]

In adjusting the illumination, it has been found convenient to pick up the light from the plane mirror with the low-power objective and center it properly before the high dry lens is swung into use. If organisms are abundant, they can usually be seen with the low power directly, and if the low- and high-power lenses are parfocal, the final adjustment is much simplified. The low-power field appears dark red, greenish, or almost black with any fluorescent particles showing as bright dots of light. The use of the immersion lens is unnecessary in routine examination of films, but it may be employed to check on the morphology of bacilli that appear atypical. Cedar oil, mineral oil, and sandalwood oil are not satisfactory as immersion media, since they all fluoresce and cast a haze over the field. Methyl salicylate (oil of wintergreen) is an excellent immersion oil with a high refractive index, and does not fluoresce. It can also be used as a mounting medium in the examination of tissue sections by the fluorescence method.

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[2] Op. cit., p. 1611.

Note that an auramine-stained film will fade in the area exposed to the ultraviolet light if left in the field for about ten minutes. Stained films fade after the lapse of time, and the brightest preparations are those which have just been made. A Ziehl-Neelsen stain can be superimposed upon an auramine stain, but not vice versa.

A photograph of the improvised light housing is shown in Fig. 391. This photograph appeared in the article by Graham.

Richards and Miller believe that concentration and examination by fluorescence affords a more delicate test than has heretofore been available and it is recommended that at least for the present it be reserved for investigational work. It should not be substituted for the present accepted, less sensitive methods, in the clinical evaluation and classification of patients, until further information is obtained as to the clinical significance of such findings.

Concentration of Sputum

(1) Antiformin

The Antiformin method is the oldest method of concentrating sputum.

To 2 c.c. of the sputum add 6.5 c.c. distilled water.

Add 1.5 c.c. of Antiformin and mix well.

Let stand for 1 to 2 hours and centrifuge.

Wash sediment with distilled water and examine under the microscope after staining by the acid-fast method.

(2) Petroff's Method

The method of concentration of the specimen of sputum given here may be used for cultivating the tubercle bacillus, for making smears, or for guinea pig inoculation.

The specimen must be fresh.

Add to the sputum specimen an equal volume of 3% sodium hydroxide (3 grams sodium hydroxide sticks to 100 c.c. distilled water).

Place a piece of heavy paper over the cork, and shake vigorously until the specimen is of a homogeneous consistency.

Place in the incubator at 37° C. for one-half to three hours to destroy the contaminating bacteria.

Remove from the incubator, and add N/1 hydrochloric acid until the specimen is neutral to litmus paper. Add the hydrochloric acid slowly, and test the reaction frequently.

Add a few more drops of the N/1 hydrochloric acid until the specimen is slightly acid.

Transfer to test tubes, and centrifuge at high speed for thirty minutes.

Decant the supernatant fluid into a container which can be sterilized, and use the sediment either for smears, for guinea pig inoculation, or for cultivation.

For cultivation, select an egg medium M-58 to M-61.

Place three drops of the sediment over the surface of each of three slants of the medium. Rub the material into the medium by means of a platinum wire. Place the cork back into the test tube. Make a small opening in the cork by puncturing it with a sterile needle.

Replace the metal cap, and place into the incubator at 37° C. for six to eight weeks. If colonies appear overnight, they are not tubercle bacilli.

Take care not to agitate the test tubes on which the inoculation has been made, since the tubercle bacillus will not grow if disturbed to a great extent.

If tubes with cotton plugs are used, flame the cotton plug after inoculation, push it into the test tube about ¼ inch from the rim, then cover with paraffin. Allow the paraffin to harden. Puncture through paraffin and cotton to allow for exchange of gases.

If there is an abundance of water of condensation on the slants, it should be removed before inoculation.

Colonies are hard, dry, wrinkled, elevated, usually yellow or pink.

(3) MacNabb[1]

To the sputum in a sterile tube add an equal amount of 3% hydrochloric acid containing 0.002% brom thymol blue.

Shake vigorously.

Allow to stand at room temperature for 2 hours.

Neutralize with 3% sodium hydroxide.

Centrifuge for 30 minutes.

Use the sediment for inoculation of infusion agar slants M-15. If growth occurs in 24 hours, treat the specimen again for 1 hour with hydrochloric acid and again neutralize.

Plant about 0.25 c.c. of the sediment on each of 4 to 6 tubes of a combination of suitable media (see below).

(4) Hanks and Associates[2]

Digestion Fluid.—

Dissolve 4 gm. sodium hydroxide
0.2 gm. potassium alum
in 95 c.c. distilled water.
Add 5 c.c. 0.4% brom thymol blue

Mix 5 c.c. of the sputum with 5 c.c. of the digestion liquid in a sterile tube.

Incubate for 30 minutes at 37° C. in the water bath.

Neutralize with 25% hydrochloric acid.

Shake for 30 minutes.

If no flocculation occurs, add 0.2 c.c. of 1% ferric chloride solution.

Centrifuge for 5 minutes.

Examine sediment, or inoculate media, or inject guinea pigs.

(5) Clorox Method

Add to the sputum an equal volume of Clorox.

Mix well.

After 3 to 5 minutes, add an equal volume of 4% sodium thiosulphate in water.

Centrifuge for 30 minutes at 3,000 r.p.m.

Examine the sediment, inoculate media, or inject guinea pigs.

Evaluation of Media for Cultivation of Mycobacterium Tuberculosis

Bovine organisms grow better on media without glycerin (M-59) or with only a small amount of this substance.

Media without dyes may be used only for the culture of uncontaminated materials or materials which have been very carefully concentrated.

The author has had excellent results with Petrik's medium (M-58) and recommends it very highly. The reviewer of this chapter has excellent results with blood medium and with charcoal media and recommends them (M-58ª and M-58ᵇ).

Regarding the other media, those of Petragnani (M-61) and Loewenstein (M-60) are of about equal usefulness.

For routine use, the inoculation of two tubes without dye, and two tubes with dye is recommended. It is advisable to prepare only two types of media.

All tubes must be incubated for 8 to 10 weeks before final reading unless they are positive, at which time they may be reported.

For animal experiments, see under *Mycobacterium tuberculosis* (pages 1480 ff., 1647).

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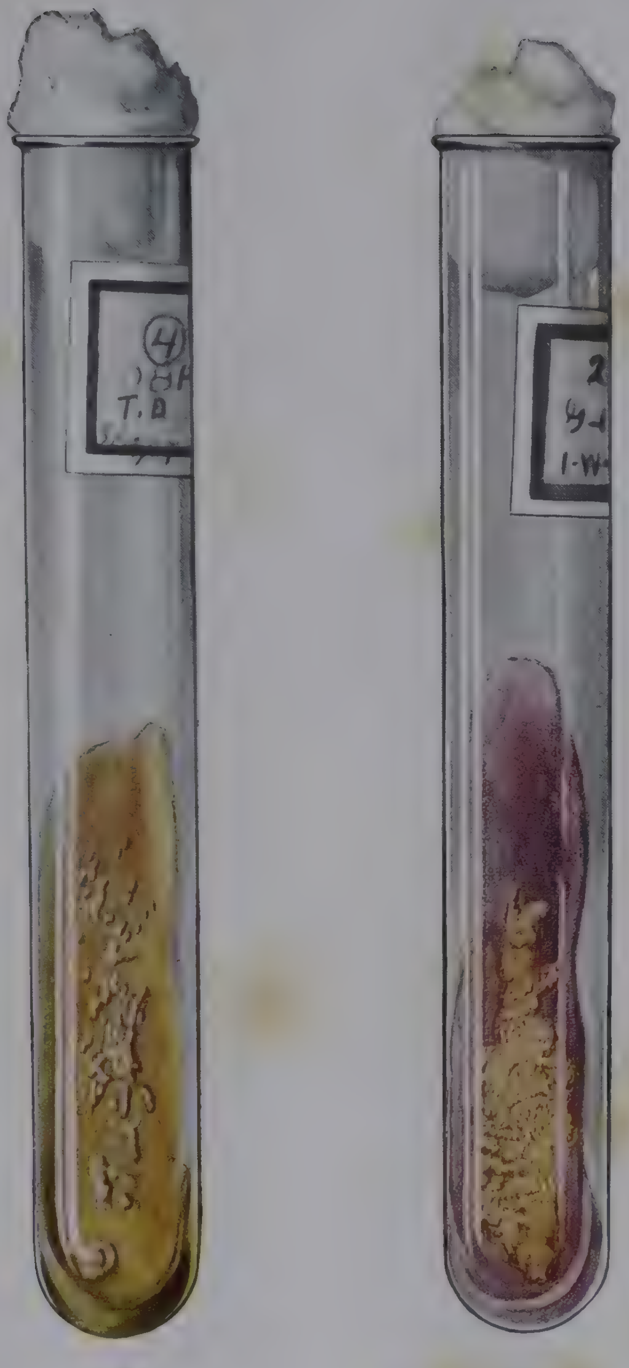
[1] Am. J. Pub. Health 26: 619, 1936.

[2] J. Lab. & Clin. Med. 23: 736, 1938.



(A) (B)

Plate XLI.—Cultures of Tubercle Bacilli: (A) on Petroff's Egg Medium; (B) on Petroff's Gentian Violet Medium.

Neutral Red Virulence Test for Mycobacterium Tuberculosis

Dubos and Middlebrook[1] described the following test for the determination of virulence of tubercle bacilli:

Take 1 loopful of culture from a solid medium, or 1 c.c. from a liquid medium.
Suspend it in 5 c.c. of 50% methyl alcohol.
Mix well and centrifuge.
Suspend again in 5 c.c. of 50% methyl alcohol.
Mix well, centrifuge, and drain.
Suspend the sediment in 5 c.c. of Dubos buffer (M-62).
Add 0.2 c.c. of a 0.05% watery solution of neutral red.
Keep at room temperature for 1 hour (not longer).
Read with a hand lens.
Virulent cultures are pink to red.

Egg Inoculation for Diagnosis of Tuberculosis

Refer to page 1475

MATERIAL FROM EAR AND MASTOID

To cultivate organisms in furuncles of the external auditory canal, the skin should be cleansed with alcohol and pus picked up with a sterile swab, using blood agar and blood agar with sodium azide (M-21, M-22) or alcohol (M-23). In *otitis media,* the otologist collects the material after disinfecting the external auditory canal. The pus should be streaked to the plates, or inoculated in tubes of enriched broth M-4 or M-6. In *acute otitis,* cultures on Loeffler blood serum M-10 or blood agar M-28, M-29 are sufficient, but in *chronic otitis,* azide or alcohol plates should be added, since the infection is generally mixed, and rapidly growing organisms may readily overgrow streptococci and similar slowly multiplying organisms. The same procedures are recommended in *mastoid infections.*

Microorganisms most frequently found are gram-positive cocci, coliforms, Protei, Pseudomonades, and diphtheria bacilli.

For differentiation, proceed as under "Pus."

Examine for *C. diphtheriae* according to the methods used for nose and throat examination.

NASAL-SINUS-NASOPHARYNGEAL SPECIMENS

In taking material from the nose for bacteriologic work, sterile swabs should be used without touching the atrium. Cultures should not be made within an hour after the application of antiseptics.

Material from infected *sinuses* should be taken by the rhinologist, under direct illumination.

In connection with the study of nasal secretions, note the work of Jacobson and Dick,[2] who found that the normal nasal flora consists chiefly of *Staphylococcus albus* and diphtheroid bacilli, with *Staphylococcus aureus* and *N. catar-*

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[1] Am. Rev. Tuberc. **58:** 698, 1948.
[2] J. A. M. A. **117:** 2222, 1941.

rhalis less frequently. They found that green-forming streptococci and pneumococci are occasional transients in the nasal cavity. The presence of streptococci, pneumococci, *Klebsiella pneumoniae,* Pfeiffer bacilli, and diphtheria bacilli indicates disease of the nasal mucosa or sinus disease or both. The work of other observers, Toerne,[2] Linton,[3] Lewis and Turner,[4] and Frankel,[5] who studied clinically normal sinuses at autopsy, proved that the sinuses are sterile in health. Frankel found that the pneumococcus was the predominant organism in sinuses which showed gross pathologic change. Oaks, Merrill, and Oaks[6] studied the bacteriology of antral washings and found one or both of the maxillary sinuses sterile in 234 of 475 patients who came to treatment because of what they presumed to be sinus infection. Jenssen[7] concluded that normal sinuses are sterile but that there are no bacteria characteristic of sinusitis. Jay[8] described a case of maxillary sinus infection in which fusiform bacilli and spirilla were isolated. Jacobson and Dick emphasize the point that the frequency with which green-forming streptococci, hemolytic streptococci, and pneumococci were isolated by culture of nasal secretion in cases of sinusitis, acute or chronic, shows the diagnostic value of this simple, inexpensive procedure.

I. Cultures From the Nasopharynx

When taking cultures from nasopharynx, use a curved wire swab that has been sterilized; avoid contamination with saliva.

Blood agar, M-28, M-29, is useful as a routine medium, also Loeffler serum M-10.

In *diphtheria,* use a sterile swab or a sterile platinum loop. Material should be immediately inoculated upon Loeffler's blood serum. The test should not be made unless some hours have elapsed after the application of an antiseptic. An attempt should be made to rub the swab deep for the reason that the diphtheria bacillus is often deeply located, while on the surface we find nothing but staphylococci. See page 1618 for the Brahdy rapid method.

To make a diagnosis of *Vincent's angina,* smears are required and the results are very definite. If interested in the diagnosis of *focal infection of the tonsil,* obtain material deep from the crypts. This material should be expressed from the crypts or secured with the aid of a special sterile glass tube attached to a suction pump. When dealing with *excised tonsils,* they should be placed in sterile gauze or saline solution immediately after removal, seared with a hot blade to sterilize the outside surface, and puncture made through the seared surface for deep inoculations.

For the examination of *meningococcus carriers,* material from the posterior wall of the nasopharynx, behind the uvula, is very useful. It is streaked to chocolate agar plates (M-31 to M-33).

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[2] Nord. med. Ark. **37:** (pt. 1) 85, 1904.
[3] Ann. Otol., Rhin. & Laryng. **39:** 779, 1930.
[4] Edinburgh M. J. **18:** 393, 1905.
[5] Virchows Arch. f. path. Anat. **143:** 42, 1896.
[6] Laryngoscope **45:** 198, 1935.
[7] Ztschr. f. Hals-, Nasen- u. Ohrenh **32:** 439, 1933.
[8] M. J. Australia **2:** 513, 1927.

II. Examination for Diphtheria

Examination for *C. diphtheriae* is carried out in the following manners:

Method No. 1

Cultures on Loeffler serum M-10 should be made from the throat, and from each side of the nose.

Incubate at 37° C. for twenty-four hours or less, preferably 12 to 18 hours.

Make smears from typical dew drop, gray colonies, and stain with Loeffler's methylene blue or other stain for granules. Look for organisms morphologically similar to the diphtheria bacillus. (Pages 1510 ff. and 1641.)

If these are found, plate out on blood agar, using the streak method of inoculation, and making the culture in such way as to give distinct colonies.

Incubate at 37° C. for twenty-four hours.

Examine the colonies microscopically to determine if they have the characteristics of the diphtheria bacillus. Margins are lacelike and irregular. Colonies are small, grayish, granular, and turn the blood brown.

Make a smear from part of one of these colonies to determine morphological similarity to diphtheria bacilli.

If the organism appears to be the diphtheria bacillus, transplant to tubes of Loeffler's blood serum medium. Make several transplants from different colonies.

Incubate at 37° C. for twenty-four hours, or until a good growth is obtained.

Make a smear and stain with Loeffler's methylene blue or other granule stain to determine whether the organism growing on the culture is the same one as that which was selected.

Add about 2 c.c. sterile physiologic saline (0.85% sodium chloride) to the slant of Loeffler's if a pure culture is obtained.

Loosen the growth from the slant by means of a platinum or nichrome loop so that the living organisms are suspended in the saline.

Inject 1 c.c. into the abdominal cavity of a guinea pig.

Tag the guinea pig. Record the number of the pig, and the laboratory identification of the culture on the record sheet.

If the organism isolated was the true diphtheria bacillus, the guinea pig will die within four days.

Diphtheria in a guinea pig is accompanied by an area of inflammation at the point of inoculation, necrosis of the tissue at the point of inoculation, and congestion in the adrenal glands so that they appear inflamed and enlarged.

If no true diphtheria bacilli are present, the guinea pig will not become ill.

For other toxicity tests, see under *C. diphtheriae*, page 1512.

Method No. 2

Collect swabs as above.

Streak to tellurite plates M-41 to M-43. The best tellurite plate for this purpose is that of Whitney and Damon (M-43). Some authors prefer other media.

Incubate for 48 hours and then pick suspicious-looking colonies.

Streak them on Loeffler slants M-10.

Inoculate guinea pigs as in Method 1 for toxicity test.

Use the growth from the Loeffler slant for biochemical tests.

Method No. 3

Brahdy Procedure

Brahdy, Brody, Lenarsky, Smith and Gaffney[1] reported their experience with the rapid culture method for the identification of diphtheria bacilli. Solé[2] stated that a description of the method had first been given by Folger at a medical meeting in Carinthia thirty-six years[2] ago but was not published. The technic is as follows:

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[1] Proc. Soc. Exper. Biol. & Med. 32: 548, 1934.

[2] Wien. klin. Wchnschr. 47: 713, 1934.

> Impregnate cotton swabs with undiluted unheated horse serum to which no preservative has been added,[3] then squeeze lightly against the sides of the tube to remove excess serum. Heat gently over a flame to obtain surface coagulation and to destroy any antibodies in the serum. Do not overheat. Keep serum-impregnated swabs in sterile test tubes. Make nose and throat cultures with these swabs in the routine manner, replacing the swabs in their sterile test tubes. Put the test tubes containing the inoculated swabs in the incubator at 37° C. for two to four hours, making smears directly from the swabs at the end of this time. The physicians' vest pocket may be used to incubate the swabs.

This method was investigated for its reliability in cases of clinical diphtheria and was found to be effective.

Diphtheria Bacillus Carriers.—There is another aspect to this work that is important, namely, the investigation of diphtheria bacillus carriers. It is well known that in carriers the culture may be positive one day and negative the next, etc. The following technic was developed at the Willard Parker Hospital, New York City:

> Transplant the four-hour rapid culture to Loeffler's serum M-10 and incubate eighteen hours. Fish colonies and examine. If diphtheria bacilli are present, emulsify the growth and inject intracutaneously into guinea pigs for virulence tests.

III. Laboratory Diagnosis of Vincent's Angina

Vincent's angina is an inflammatory condition in the mouth, pharynx, or throat, often seen upon the tonsils. It frequently simulates diphtheria. Later, ulcers appear. This disease yields to local treatment.

Plaut[4] and Vincent[5] reported the bacteriologic findings in these cases. They showed in smears from the lesions a spindle-shaped or fusiform bacillus, often associated with the spirillum of relapsing fever. These fusiform bacilli described by Vincent and others are from 3 to 10 microns in length. They taper gradually toward the ends, ending in blunt or sharp points. They do not readily stain except with Loeffler's methylene blue, carbol fuchsin, or Giemsa stain. This organism has been listed as *Fusobacterium plauti-vincenti.* Weinberg, Nativelle and Prévot make a distinction between *Fusobacillus plauti* and *Fusiformis fusiformis* (Vincent), the former being actively motile and nonpathogenic and the latter nonmotile and pathogenic. The spirilla are longer than the fusiform bacilli, with shallow and irregular undulations. They do not stain well. There has been considerable discussion as to whether or not fusiform bacilli develop into spirochetes and spirochetes into fusiform bacilli. It seems from the weight of opinion that these fusiform bacilli do not develop into well-defined spirochetes. Evidently a spiral form by some of the fusiform bacilli produces loosely coiled spirochete-like organisms which are classified morphologically as a species of Borrelia.

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[2] Op. cit., p. 1617.

[3] Horse serum saturated with chloroform as a preservative may be used.

[4] Deutsche med. Wchnschr. **20**: 920, 1894.

[5] Ann. de l'Inst. Pasteur **10**: 488, 1896.

Technic of Examination.—

Smears should be made from the ulcers or pus from the lesions on glass slides. Diagnosis is made from smears from the gums. Later the lesions spread over the entire mucosa of the mouth. Fix in the flame.

Stain one slide with dilute carbol fuchsin for about thirty to sixty seconds. Stain the other with Loeffler's methylene blue for 1½ to 3 minutes.

Wash with distilled water. Allow to dry in the air.

Examine for *Borrelia vincentii* and fusiform bacilli. The *Fusobacterium plauti-vincenti* is sharp-pointed at both ends. It stains gram-negative. Stain is irregular. It is a normal inhabitant of the mouth, and a secondary factor in the disease. Growth occurs on serum-containing media under strict anaerobic conditions.

The borrelia are found best in the dark-field.

If found in large numbers, report *Borrelia vincentii* and *Fusobacterium plauti-vincenti* found. This is a positive report.

A negative report may read: No evidence of Vincent's angina; or no borrelia or fusiform bacilli found.

IV. Beta-Hemolytic Streptococci

For beta-hemolytic Streptococci, streak a blood agar plate M-28, M-29. Incubate 24 hours and observe for typical colonies.

Recommended Procedure.—

Make direct smears and stain for Plaut-Vincent organisms.

Inoculate a Loeffler tube M-10 *or* a reliable tellurite plate M-41 to M-43.

Streak a blood agar plate M-28, M-29.

Proceed as above.

V. Pneumococci

In children, who swallow their sputum, the pharyngeal swab may help to isolate pneumococci.

Make streak inoculation from the swab on blood agar plate M-28, M-29, and select isolated colonies for further plating or mouse inoculation.

VI. Meningococci

The following procedure is highly recommended:

The original cultures are made either on a slant of Loeffler's blood serum medium M-10, or on plates with chocolate agar (M-31 to M-33).

Cultures should be made from the throat, and from each side of the nose. It is better in the case of the cultures from the nose to insert the swab into the nose from the back rather than to make a culture from the nasal secretions.

The plates, or the slants, should be incubated at 37° C. for twenty-four hours.

Select typical colonies, make smears, and stain with the Gram stain. If there are any typically shaped organisms, proceed with biochemical examination (page 1501).

The gram-negative diplococci which may be found in the nose and throat, with the exception of the *Neisseria catarrhalis*, all ferment levulose. The meningococcus ferments maltose, but the catarrhalis does not.

After biochemical identification, perform serologic tests as described on page 1502.

VII. Pertussis

Bradford and Slavin* devised a method for collecting suitable material from the nasopharynx by using a thin, flexible copper wire with a bit of cotton wrapped around the end. This is introduced into the nasopharynx through the nose.

Inoculate a plate of Bordet-Gengou (M-39) medium, preferably with penicillin (M-40), with the material.

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*Proc. Soc. Exper. Biol. & Med. 43: 590, 1940.

VIII. Tuberculosis

A laryngeal swab on a long wire is passed into the larynx, under the control of a laryngoscope. The patient is instructed to cough.

The swab is withdrawn, taken off the wire with a pair of sterile forceps, and placed in some saline.

Use the material so collected for culture or animal inoculation.

IX. Leprosy

Swabs taken from the deep parts of the nasal passages are used to make smears for acid-fast staining.

COUGH PLATE

Hold the plate containing Bordet and Gengou medium (M-39 or M-41) a few inches in front of the patient's mouth and induce him to cough on the plate. Incubate the plate aerobically at 37° C. for four or five days. To prevent drying of the medium, keep the plates in a moist atmosphere. This may be accomplished by placing the plates on a piece of heavy wire mesh or perforated metal over a flat container of water, keeping the plates covered with an inverted glass vessel.

Colonies of *Hemophilus pertussis* usually appear in five days. They are small, gray-white, hemispherical, opaque, "mercury droplets." The quantity of blood in the medium is too great to make the reading of the zone of hemolysis distinct enough to be of diagnostic value.

Digestion of the hemoglobin will be noted.

Serologic Test:

Powell and Jamieson[1] reported their results with a simple test of agglutination for the diagnosis of pertussis. This test was suggested by the typhus bedside test of Castañeda, Silva, and Monnier.[2] The test can be carried out with a drop of blood from the patient's finger or ear, or with a drop of serum if the blood is drawn and sent to a central laboratory.

Antigen.—The antigen consists of a homogeneous suspension in sodium citrate solution of pertussis bacilli standardized against strongly positive, weakly positive, and negative agglutinating sera and usually adjusted to a turbidity of about 100 billion bacilli per c.c., colored to a desired intensity with methylene blue, and preserved with 1:10,000 Merthiolate. This antigen may be kept in capillary tubes similar to those used to dispense smallpox vaccine, or it may be dispensed in small dropper bottles. A capillary tube of antigen (approximately $\frac{1}{40}$ c.c.) is sufficient for one drop test, and multiple tests may be done, each with a single drop from a bulk dropper bottle. With proper care and cleanliness, the antigen in dropper bottles will not readily become contaminated in the presence of the merthiolate preservative.

Agglutinating Antibody.—This may be tested for in a single small drop of blood from the finger or ear as obtained with a Bard-Parker blade for blood count, or a similar drop of serum or citrated blood if the blood is sent to a central laboratory.

Technic.—

Place a small drop of the blue pertussis antigen comprising approximately $\frac{1}{40}$ c.c. on a piece of hard surface cardboard or stiff paper of the size of a microscope slide and not readily wetted through. With match stick, toothpick, or probe, quickly spread the drop of antigen to form a circle about one-half inch in diameter.

Add a similar drop of blood or serum to the drop of antigen, stir the mixture a few seconds, and then rock or tilt the cardboard or paper, holding the drops, back and forth for a minute.

The sodium citrate in the antigen-suspension prevents coagulation of the blood.

Reactions.—

A *positive* result is readily observed as granulation or clumping of the blue antigen. If blood is used, read against a reddish background; if serum is used, read against a white background. Too long agitation of the test-drop mixture giving a positive reaction may cause the clumped antigen to form a blue ring at the margin of the drop. A *negative* reaction shows a diffuse blue color modified with red if blood is used.

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[1] J. Immunol. 43: 13, 1942.

[2] Rev. Med. Hosp. Gen. 2: 382.

Following agitation and observation of the antigen-blood mixture for about one minute, set aside the drop-mixture on cards or paper and allow to dry without disturbing. After partial drying has taken place, additional agitation may produce artefacts in the negative reactions.

The entire test may be conducted and read in two or three minutes, since only one minute's agitation suffices for the incubation period. The test card or paper holding the drop-mixture, after drying, comprises a permanent record which may be glued to the patient's record, or the original test may be done directly on a blank area of the record, if this is not quickly wetted through.

TEETH

(a) To make a bacteriologic examination of an extracted tooth, proceed as follows:

1. Wall off the operative field with sterile cotton rolls.
2. Dry off the area about the tooth with sterile gauze and apply 3 per cent tincture of iodine. Allow it to penetrate well into the gingival margin about the tooth.
3. Extract, and while holding the tooth in the extraction forceps, sever the apex with a pair of cutting forceps and drop it untouched into a sterile test tube or vial.
4. Cultivate this fragment in a rich broth medium like brain broth (M-4) or drop the fragment into a sterile screw-top vial containing a small amount of sterile sand and gelatin-Locke solution. Shake for ten minutes to macerate the tissue and transfer the material with a sterile pipette to tubes of glucose-brain broth and streak on blood agar plates.

(b) Cultures of the socket can be made by:

1. Walling off the tooth with particular care with sterile cotton rolls to prevent contamination with saliva or the tongue.
2. Sterilizing the gums with tincture of iodine.
3. Extracting with forceps with sterilized beaks.
4. Curetting with sterile curet or with a sterile cotton swab removing material for inoculation in glucose hormone broth or on blood agar plates.

(c) To culture the periapical region through the root canals with the tooth in position, proceed as follows:

1. Isolate the tooth with rubber dam.
2. Sterilize the coronal surface with a 3 per cent tincture of iodine.
3. Remove the filling with sterile instruments.
4. Remove filling in the root canal with sterile instruments.
5. Cleanse and dry the canal with sterile cotton and insert sterile paper points slightly moistened with sterile saline solution to absorb any moisture oozing into the canal; remove the points and drop them into a tube of glucose hormone broth.
6. If no moisture oozes in, pass a sterile, fine broach or pick through the canal and drop it into a tube of the medium.

Smears may be made from the gums. Allow to dry and stain for spirochetes and bacteria.

Lactobacilli are grown on L medium (M-114).

SKIN

1. The examination for *meningococci* and *Rickettsiae* is possible from material taken from petechiae.

2. To make smears for *Mycobacterium leprae,* make a cut into the thickened skin with a razor blade. Turn the blade 90 degrees and collect scrapings from the middle of the deepest point of the cut. Stain with acid-fast methods. Examine also for foam cells.

3. *Mycologic examination by culture.*—A good all-round method is to plate contaminated material on mycophil medium (M-106), blood agar (M-28, M-29), and Littman medium (M-105).

All mycotic material should be incubated at room temperature at 37° C., and under anaerobic conditions as 37° C. At least two plates should be used for each incubation. Plates which are not incubated anaerobically must be sealed with Plasticine, adhesive tape, or other material, to prevent evaporation.

The plates must be incubated for at least 3 weeks.

When colonies of molds or yeasts appear, they must be obtained in pure culture by transferring them to fresh plates. Wort agar M-101 and malt extract agar M-100 are frequently used as differentiating media. Fermentation reactions and growth in broth must also be observed.

For details, see the section on mycology, Chapter XI.

EYE EXAMINATION

Generally speaking, the bacteriologic causes of *ocular* diseases are best found during the stage of the disease when the disease is developing and not in the stage of retrogression. In *conjunctivitis,* avoid taking material which has been in contact with the angles or margins of the lids. It is best to evert the lids and take the inoculation with a sterile platinum loop from inside. In dealing with infections of the cornea, the greatest care must be exercised to avoid injury to the tissue. Anesthetize the cornea and use the point of a sterile Graefe knife or needle if the organism is likely to be deeply located.

When looking for bacteria in the anterior chamber, the specimen is obtained by an ophthalmologist himself with a small syringe and needle. In iridectomies, a piece of the iris should be placed in chopped meat medium M-12, with a pH 7.4 to 7.6, suitable for the cultivation of streptococci and pneumococci.

In general, do not make smears or cultures within three or four hours after the use of an antiseptic solution. In attempting to make bacteriologic studies from enucleated eyes, the outside of the eye should be seared with a hot iron and openings made through this sterilized point.

Laboratory Diagnosis of Eye Affections*

Unlike other mucous membranes of the body, the conjunctiva has a limited normal bacterial flora, the only two constant inhabitants being *Corynebacterium xerose* and *Staphylococcus epidermidis,* both of which are saprophytes. The mechanical cleansing action of the tears as well as their lysozyme content act to eliminate accidental contaminants, usually air bacteria, as well as to inhibit the normal conjunctival flora. In the presence of an acute or subacute conjunctivitis the saprophytes are often crowded out by the invading microorganisms to such an extent that in secretion films or epithelial scrapings the exciting agent is found in pure or almost pure culture. It is this feature which renders the film diagnosis of conjunctivitis a practical and often simple procedure.

The Importance of an Etiological Diagnosis

The determination of etiology, when possible, is a fundamental principle of scientific medicine. The importance of knowing the active microbic agent in conjunctivitis is obvious for epidemiologic, diagnostic, and prognostic purposes, and proper management is often possible only when the etiology is known. In the stress of private and clinic practice, however, a bacteriologic diagnosis is often neglected. A technic adequate for diagnostic purposes but simple enough to require a minimum amount of time and effort follows. The film technic is designed particularly for the ophthalmologist in private practice while the combined film and culture technic is designed for the clinic.

Film Technic

Material Required.—Two stains are necessary. The Gram stain (see page 1409) gives all information needed in conjunctivitis of bacterial origin. Giemsa stain gives the information required in trachoma and in inclusion conjunctivitis (inclusion blennorrhea and swimming-pool conjunctivitis), and also enables the eosinophilia of vernal catarrh to be determined.

Giemsa Stain.—Fix scrapings (on a glass slide) in methyl alcohol for ten minutes. Stain overnight in the incubator at 37° C. with Giemsa stain diluted 1 drop to each c.c. of neutral distilled water. Decolorize in 95% alcohol twice for a total decolorizing time of five seconds. Dry in the air and examine.

If rapid diagnosis is required, the following technic may be used: Fix one minute with Wright stain. Dilute the fixative with an equal number of drops of neutral distilled water and allow the solution to remain for four minutes. Transfer to dilute Giemsa and stain for fifteen minutes or longer. Decolorize in alcohol. Dry in the air.

Method.—Make four preparations from each case, two epithelial scrapings, two secretion films. Place one epithelial scraping and one secretion smear in absolute methyl alcohol. Stain secretion film with Gram stain. If it gives satisfactory information, discard the other slides.

Culture Technic

Media.—Use blood agar plates M-28, M-29 routinely. Loeffler serum (M-10) and chocolate agar (M-31 to M-33) should be available.

Method.—Cultures are taken most satisfactorily from the lower cul-de-sac, with care to avoid the lashes and lid margin. In chronic blepharo-conjunctivitis, take cultures also from the lid margins and the inner canthus. Incubate at 37° C.

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*We are indebted to Dr. Phillips Thygeson, of the Department of Ophthalmology, Columbia University College of Physicians and Surgeons, New York, for much of the information on this subject.

The Conjunctival Bacteria

The morphologic and biologic characteristics the determination of which is essential for the diagnosis of the conjunctival bacteria are outlined in Tables 162 and 163. Other characteristics are enumerated below.

Saprophytes.—

Corynebacterium xerose (xerosis bacillus). Often found in large numbers on degenerated epithelial cells. Colonies on blood agar are characteristically small, grayish white, dry, granular, and slow-growing. Barred and granular forms of the bacterium usually occur in specimens from culture but are often absent in direct smears.

TABLE 162.—DIFFERENTIAL DIAGNOSIS BY CULTURE OF THE COMMON CONJUNCTIVAL BACTERIA

- COCCI
  - GRAM-POSITIVE
    - CLUSTERS—STAPHYLOCOCCUS—
      - Golden pigment. Beta-hemolysis. Coagulase positive. Pathogenic. *STAPHYLOCOCCUS AUREUS*
      - Same except no pigment. *STAPHYLOCOCCUS ALBUS*
      - No pigment. No hemolysis. Coagulase negative. Pathogenic. *STAPHYLOCOCCUS EPIDERMIDIS*
    - CHAINS
      - Blisterlike colony with central depression. Alpha hemolysis. Bile soluble. Ferments Inulin. *D. PNEUMONIAE*
      - Minute colony with opaque heaped-up center. Insoluble in bile. Does not ferment inulin.
        - Beta (clear hemolysis) *STREPT. HEMOLYTICUS*
        - Alpha Hemolysis. *STREPT. VIRIDANS*
        - No Hemolysis *STREPT. NONHEMOLYTICUS*
    - PACKETS *SARCINA*
  - GRAM-NEGATIVE
    - Scanty or no growth on ordinary media. Good growth on chocolate agar. Ferments dextrose, not maltose. Grayish opalescent colony. Oxidase positive *N. GONORRHOEAE*
    - Good growth on glucose blood agar, chocolate agar. Ferments dextrose and maltose. *N. (MENINGITIDIS) INTRACELLULARIS*
    - Good growth on plain agar. Does not ferment dextrose nor maltose. *N. CATARRHALIS*

Corynebacterium pseudodiphtheriticum (Hoffman's bacillus). Typically shorter and fatter than the xerosis bacillus. Not barred nor granular. Colonies on blood agar are large, opaque, and granular.

Staphylococcus epidermidis (Staphylococcus albus). Large, china-white colonies. No hemolysis.

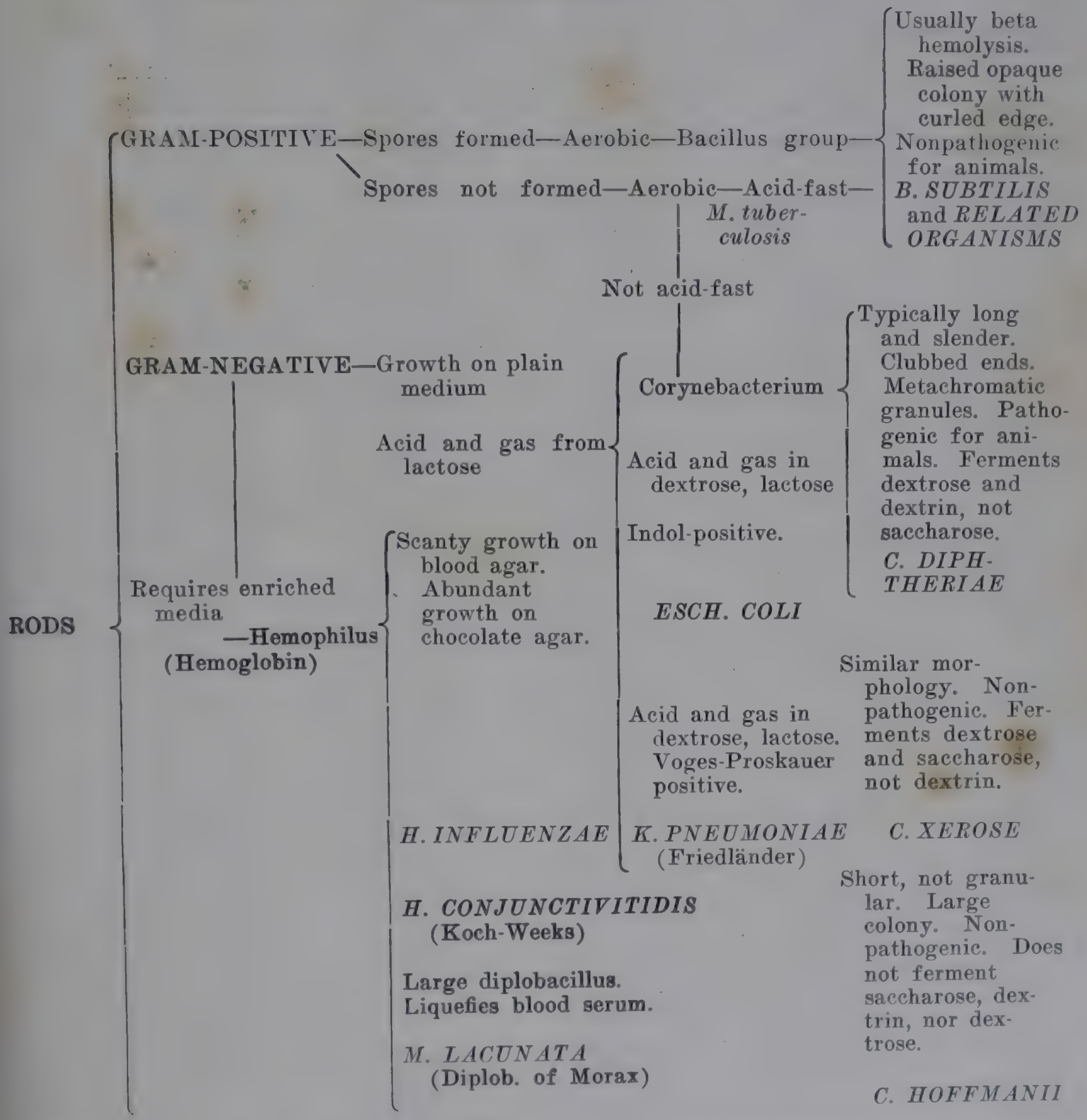
Sarcinae. Air bacteria. Colonies large, granular, and usually pigmented.

Pathogens.—

Neisseria gonorrhoeae (gonococcus). See pages 1497, 1640, for culture methods.

Hemophilus conjunctivitidis (Koch-Weeks bacillus). Produces typically an acute conjunctivitis with marked bulbar injection. Epithelial scrapings usually show turflike arrangement of the bacteria on the cells. The essential morphologic characteristics are minute size and slender bacillary shape.

TABLE 162—CONT'D



- RODS
  - GRAM-POSITIVE
    - Spores formed—Aerobic—Bacillus group—
      - Usually beta hemolysis. Raised opaque colony with curled edge. Nonpathogenic for animals. *B. SUBTILIS* and *RELATED ORGANISMS*
    - Spores not formed—Aerobic—Acid-fast—
      - *M. tuberculosis*
      - Not acid-fast
        - Corynebacterium
          - Typically long and slender. Clubbed ends. Metachromatic granules. Pathogenic for animals. Ferments dextrose and dextrin, not saccharose. *C. DIPHTHERIAE*
          - Similar morphology. Nonpathogenic. Ferments dextrose and saccharose, not dextrin. *C. XEROSE*
          - Short, not granular. Large colony. Nonpathogenic. Does not ferment saccharose, dextrin, nor dextrose. *C. HOFFMANII*
  - GRAM-NEGATIVE—Growth on plain medium
    - Acid and gas from lactose
      - Acid and gas in dextrose, lactose. Indol-positive. *ESCH. COLI*
      - Acid and gas in dextrose, lactose. Voges-Proskauer positive. *K. PNEUMONIAE* (Friedländer)
    - Requires enriched media —**Hemophilus** **(Hemoglobin)**
      - Scanty growth on blood agar. Abundant growth on chocolate agar.
        - *H. INFLUENZAE*
        - ***H. CONJUNCTIVITIDIS*** **(Koch-Weeks)**
      - **Large diplobacillus. Liquefies blood serum.** *M. LACUNATA* **(Diplob. of Morax)**

Hemophilus influenzae (influenza bacillus). Produces typically a subacute conjunctivitis with bulbar injection. Secretion films show minute cocco-bacilli, often paired, occasionally in short chains. Some individuals, except for their reaction to the Gram stain, could be confused with small pneumococci. The cultural characteristics are identical with those of Koch-Weeks bacillus and the morphology in direct smears is the only means of distinguishing between the two microorganisms.

Many bacteriologists[2] believe that *H. conjunctivitidis* and *H. influenzae* are identical. The morphologic differences between them, however, though lost in culture, are striking in direct secretion preparations. On the basis of this differentiation, *H. conjunctivitidis* produces typically an acute conjunctivitis while *H. influenzae* is almost invariably associated with a subacute disease. In support of this conclusion, and indicating that the geographical distribution of the two bacteria is not the same, Thygeson observed in Iowa City that mild conjunctivitides are associated with influenzal infections of the nose and throat, while the severe infections are almost invariably pneumococcic. Koch-Weeks conjunctivitis, according to his diagnosis, is almost never seen.

Diplococcus pneumoniae (pneumococcus). Capsules are usually not demonstrable in pneumococci from the conjunctiva. Saprophytic forms are usually not bile soluble, but this property can be readily restored by mouse passage.

Table 163.—Differential Diagnosis by Morphology of the Common Conjunctival Bacteria

COCCI	GRAM-POSITIVE	Clumps or Pairs. Pleomorphic. Never in live epithelial cells—*STAPHYLOCOCCUS.* Pairs or short chains—*STREPTOCOCCUS.* Lancet-shaped diplococci—*D. PNEUMONIAE.*
	GRAM-NEGATIVE	Kidney-shaped diplococci—*N. GONORRHOEAE.* Parasitic in live epithelial cells.
RODS	GRAM-POSITIVE	Pleomorphic-clubbed ends—Growth on live epithelial cells—*C. DIPHTHERIAE.* Similar morphology—Not parasitic on live epithelial cells—*C. XEROSE.*
	GRAM-NEGATIVE	Encapsulated—*KLEBSIELLA PNEUMONIAE (FRIEDLAENDER).* Large diplobacillus—*M. LACUNATA* (Diplobacillus of Morax). Minute coccobacillus—*H. INFLUENZAE.* Minute slender rod—*H. CONJUNCTIVITIDIS* (KOCH-WEEKS).

Streptococcus pyogenes. Rarely produces a catarrhal conjunctivitis and exhibits no affinity for the conjunctival epithelium. It is probably the cause of the majority of the cases of chronic pseudomembranous conjunctivitis which are not diphtheritic. The streptococcic type is really a subconjunctivitis and the bacteria are usually demonstrable only when the epithelium is broken.[10]

Corynebacterium diphtheriae (diphtheria bacillus) produces typically a pseudomembranous conjunctivitis. Scrapings for direct microscopic examination and for culture should be taken after removal of a portion of the membrane. The finding of pleomorphic gram-positive rods on living epithelial cells is diagnostic. Diagnosis by culture on Loeffler serum is obtainable in from twelve to eighteen hours.

Staphylococcus aureus: a common cause of subacute conjunctivitis in the newborn and chronic conjunctivitis in the adult. Pathogenic strains generally show wide areas of beta hemolysis on blood agar. Strains vary widely in amount of pigment produced.

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[1] McClung, C. E.: Handbook of Microscopical Technique, New York, 1929, Paul B. Hoeber inc., p. 93.

[2] Durand, P., and Lumbroso, U.: Arch. l'Inst. Pasteur Tunis 15: 71, 1926.

Moraxella lacunata (Diplobacillus of Morax) produces typically a chronic blepharo-conjunctivitis. A gram-negative, large diplobacillus which does not grow on plain medium, but which actively liquefies Loeffler serum, may be considered to be the *Diplobacillus of Morax.*

Special Considerations

Lacrimal Conjunctivitis.—This type of conjunctivitis is secondary to infection of the lacrimal sac and does not respond to treatment of the conjunctiva. The prognosis is most favorable in the newborn in whom the lacrimal stenosis usually heals spontaneously. In adults the condition usually requires either removal of the sac or one of the more physiologic operations which restore drainage into the nose. The chronic conjunctivitis which follows dacryocystectomy is usually nonbacterial and probably results from the disturbance in lacrimal function.

The commonest agents in lacrimal conjunctivitis are, in order of frequency: *D. pneumoniae, H. influenzae,* and *Str. pyogenes.*

Vernal Catarrh.—The constant finding of eosinophilic cells in the conjunctival secretion is of diagnostic importance. The eosinophiles are typically fragmented and the eosinophilic granules widely scattered. The finding of scattered eosinophilic granules, even though but few cells are present, is extremely suggestive of vernal catarrh and is particularly useful in doubtful cases in which the clinical signs of the disease are not fully developed. A number of subclinical cases which have the subjective but not the objective signs of the disease yield positive microscopic findings. Scrapings from the upper tarsal conjunctiva are the most instructive.

Trachoma Bodies and Other Inclusion Bodies

See pages 1736 ff.

GASTRIC CONTENTS

The most important bacteriologic examination of gastric contents is that for tubercle bacilli.

The examination of the gastric contents for tubercle bacilli frequently gives positive evidence that the minimal infiltration of lungs is pathologically active and in need of treatment. This procedure is of great value in helping to determine, especially in the asymptomatic cases, how long active treatment should be continued. According to Stiehm,[1] this examination revealed positive evidence of tuberculosis in 71 per cent of the cases recorded by him. He considers that one of the criteria of a healed lesion is the repeated absence of tubercle bacilli in the gastric contents as indicated by guinea pig inoculation. Strict adherence to this principle should result in fewer recurrences in the patients with apparent but not real cures.

Armand-Delille and Vibert reported on this procedure in 1927 and in the course of the following nine years Armand-Delille and Kérambrun investigated 1,298 cases of respiratory disease in children by this method. Of these, 712 were exposed to tuberculosis in their homes and manifested signs of pulmonary or lymph node tuberculosis and positive cutaneous reactions. The gastric contents were examined by direct microscopy and in some instances by inoculation into guinea pigs or by the culture method of Loewenstein or of Petragnani. Bacilli were recovered in more than 50 per cent of 668 tuberculous children. The method demonstrated the possibility of discovering bacillus carriers in children presenting no more than tracheobronchial adenopathy.

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[1] Am. J. M. Sc. **194**: 340, 1937.

Poulsen and his co-workers[2] in Denmark examined 622 tuberculous children and found 199 positive for tubercle bacilli by guinea pig inoculation of gastric contents. Nils Levin[3] of Sweden examined 283 children with active primary tuberculosis and found that 68 per cent had tubercle bacilli in their gastric contents. Only a few were positive on direct smear. Gourley[4] reported the results of studying a series of 210 tuberculous children by means of gastric lavage and guinea pig inoculation. Tubercle bacilli were demonstrated in 40 per cent. Stadnichenko and Cohen[5] reported their results of using the method on 600 adult patients admitted to the Chicago Municipal Tuberculosis Sanitarium. Examination of the patients with clinical tuberculosis who had never shown a positive sputum revealed the presence of tubercle bacilli in 46 per cent when gastric sediment was injected in guinea pigs; 39 per cent of these were positive in smears.

It is generally believed that inoculation into guinea pigs of centrifuged fasting gastric contents is undoubtedly the most sensitive method for the diagnosis of pulmonary tuberculosis.

Technic.—

The method of examination, as advised by Stiehm,[6] is as follows:

Centrifuge the specimen for one hour at 2,000 r.p.m. Pour off the supernatant fluid and make smears directly from the sediment; stain for acid-fast bacilli. Mix the remainder of the sediment thoroughly with an equal quantity of 10% (by weight) sulphuric acid and allow to stand for 45 minutes with occasional shaking. Neutralize with 8% potassium hydroxide using brom thymol blue as the indicator. After neutralization again centrifuge the specimen, pour away the supernatant fluid, and divide the sediment into two equal parts. One part is used for cultures and the other for guinea pig inoculation. The cultures are made by inoculating each of two tubes of egg medium M-59 with 0.5 c.c. of the sediment. Stopper the culture tubes with corks and incubate at 37° C. for 3 to 4 weeks. Inject the remaining half of the sediment intraperitoneally into a guinea pig; watch for a period of six weeks. At the end of this time, kill the guinea pig and examine for gross evidence of tuberculosis. If gross lesions are found, confirm the diagnosis of tuberculosis by the presence of acid-fast bacilli in stained smears made either from an enlarged lymph node or the spleen.

To prevent the possibility of outside contamination, sterilize the Rehfuss tube, syringe, and bottle used in obtaining the specimen by boiling. Prepare the reagent solutions with sterile distilled water, and use sterile pipettes.

Another technic which is used at Mount St. Rose Sanatorium in St. Louis, Missouri, is that advised by Henske,[7] as follows:

To the specimen of sputum or stomach washing from a fasting stomach, add an equal portion of 10% Antiformin solution. Allow to stand 24 hours.

Centrifuge at 2,000 r.p.m. for 30 minutes. Pour off supernatant fluid and make smear from residue.

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[2] Am. J. Dis. Child. 47: 307, 1934.

[3] Acta paediat. 17: (Supp. 1) 160, 1935.

[4] Nat. Tuberc. Assoc., 33rd annual meeting, 1937, p. 131.

[5] Nat. Tuberc. Assoc., 33rd annual meeting, 1937, p. 125.

[6] Loc. cit. (Stiehm).

[7] Henske, A. C.: Personal communication.

Stain with Ziehl-Neelsen stain. Examine 24-hour sputum specimens for ten consecutive days or three days of stomach washing.

If smears fail to reveal the presence of acid-fast organisms, then inoculate guinea pig. The percentage of positive findings with this technic is far greater than with the ordinary sputum examinations and is employed at Mount St. Rose Sanatorium as a final procedure before discharging a patient as an arrested case.

Most laboratories use the media of Loewenstein (M-60) and Petragnani (M-61) for the culture of concentrated gastric washings.

Gastric contents may be examined for lactic bacilli. The so-called Boas-Oppler organism belongs to this group.

Streak with the centrifuged gastric contents 1 or 2 L agar plates (M-114). Incubate and observe for typical colonies.

BILE

The bile should be sent to the laboratory in a sterile container.

Plate out the bile on blood agar, M-28, 29.

At the same time, make inoculations to an eosin-methylene blue plate (M-71, M-72).

Incubate for twenty-four to forty-eight hours, or until a growth is obtained.

Proceed in the usual manner for differentiation of the bacteria present.

If a search for typhoid bacilli is to be made, proceed in the manner described under Salmonella.

Report the organism isolated.

STOOL EXAMINATION

I. Collection of Material

Stool examinations are not complete if they do not include both bacteriologic and parasitologic studies.

Only fresh or properly preserved material may be used. Many pathogenic organisms, such as, Endamoebae, Shigellae, etc., can be recovered from specimens only if the specimens have been cultured or fixed within a few minutes after the stool has been passed.

Both stool and fecal material collected from the intestines may be examined.

(a) Fresh Stool Specimens.—

In hospitals the nurses usually collect fresh stool specimens and deliver them immediately to the laboratory. In clinical laboratories the technician is often responsible for the collection of the stools. The patient is asked to come to the laboratory in the morning, and instructed to collect the stool on a cardboard tray fitted into the toilet.

Or, the patient may be given a sputum collecting waxed-paper box and wooden spatulas and instructed to collect the specimen in this container. Some laboratories supply the patients with one-half pint ice cream cardboard cartons or wide-necked glass bottles, and ask the patients to obtain the specimens directly in these.

(b) Post-Cathartic Stool Specimens.—

Instruct the patient to take one to three teaspoonfuls of sodium sulphate in water before breakfast. Collect material from the second or third bowel movement. Do not give oil as a cathartic. If permanent hematoxylin stained slides are to be made for examination for protozoa, glycerin suppositories are admissible, principally for children.

The laws in many states require use of post-cathartic stools for examination for carriers of Salmonellae.

(c) Fecal Specimens.—

Material for fecal specimens is collected by the proctologist.

A method of obtaining a swab of material for bacteriologic examinations, which is very practical and useful, is that advocated by Hardy, Watt, and DeCapito.[1] Their method is to introduce a small lubricated rubber tube into the anus. Before introducing it, a dry swab is placed within the lumen of the lubricated tube. The necessary materials are inexpensive and readily available. Gum rubber tubing (0.5 cm. inside and 0.8 cm. outside diameter) is purchased in bulk and cut into 12 cm. lengths. One end is beveled for about 1 cm. Cotton swabs are prepared on the usual wooden applicators, but the cotton must be wound tightly, the end covered completely, and it must pass readily into the lumen of the tube. For use, the swab is placed in the rubber tube with its tip slightly short of the beginning of the beveled opening. The external surface of this end of the tube is lubricated. The jelly should not reach the swab nor cover the opening. With patient in convenient position the unit is very easily inserted past the sphincter and up about one-half the length of the tube. The swab is exposed by withdrawing the tube 2 to 3 cm. The specimen is collected by rotating the applicator while sweeping it in a circular motion. The swab is then drawn back into the tube and in this position removed from the patient. The rubber tube and swab are separated and the latter is immediately used for plating. Later the tubes are boiled, washed, sterilized, and stored for future use. One precaution must be observed in collecting specimens from individuals with a watery diarrhea; the rubber tube must be compressed between the fingers to prevent an undesired discharge of fecal material.

This method is especially advised for hospitalized cases and for use in the studies of men in military barracks. Nurses may be permitted to collect specimens in this way from individuals ill at home.

II. Preserving Fluids

(a) For Bacteriologic Examinations.—

Prepare preserving fluids in 30 c.c. screw-capped bottles. Put about 10 c.c. of the preserving fluid in each bottle. Put the inoculum, consisting of 1 to 2 gm. of the fecal material, or of the end of the swab on which the rectal material was collected, into the bottle.

Mix the stools thoroughly with the preserving fluid and send to the laboratory.

The best general preservative is buffered glycerol-saline formula M-96. As yet no reliable medium is known for Shigellae.

If the specimen is to be carried only a short distance, from the proctologist's office to the laboratory, both in the same building, put the swab in peptone broth formula M-1 or M-2.

Fecal material must never be permitted to dry before plating.

(b) For Parasitologic Examination.—

Use the proctologic swab or the Hardy swab directly to make smears for permanent hematoxylin stained preparations. *Under no conditions must the material dry on the swab or on the slide.* Drying precludes the finding of amebae. If such material is to be preserved, fill a Koplin jar with Schaudinn's fluid, and ask the proctologist to prepare smears on slides, place them in the Schaudinn fluid, and send the jars back to the laboratory for staining and examination.

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[1] Pub. Health Rep. 57: 521, 1942.

When larger amounts of stools are to be transported (in examination for eggs of parasites, etc.) mix about 1 to 2 gm. of the feces with 10 c.c. of 10% Formalin in a 30 c.c. screw-capped bottle and mail to the laboratory.

Details on the parasitologic examination of feces are given in Chapter XII.

III. Plating

Little can be learned from examining a Gram-stained slide made from the stools. Gram-negative organisms and sporeformers are always present. No importance is attributed to the so-called "milk reaction." For this reaction sterile milk is inoculated with the stool and incubated. If "stormy" fermentation is present, *Cl. perfringens* (*welchii*) is reported. At least twenty organisms in addition to *Cl. perfringens* cause stormy fermentation of milk, and may be found in the intestines. Scientific basis for this test is, therefore, lacking. *Cl. perfringens* does not seem to be pathogenic in the normal bowel, except type F.

It is not possible to determine from the clinical picture whether Shigellae alone or Salmonellae should be looked for. The methods presented here are therefore directed to finding members of both genera.

Both enrichment fluids and plates are used. The enrichment fluids serve primarily to facilitate isolation of Salmonellae, including the typhoid bacillus. Of the plating media, those with bismuth sulphite, formulas M-69 and M-70, and brilliant green-phenol red agar M-73, while highly selective for Salmonellae, do not permit isolation of Shigellae. Shigellae require nonselective media, such as desoxycholate agar M-64, eosin-methylene blue agar M-71, M-72, etc. Of the media of average selectivity, SS agar M-67 is most popular.

In the methods presented here, various suggestions are offered for laboratories of different equipment and size. Small establishments require simpler methods; fewer plates are used. There is, however, no possibility of using fewer plates than indicated if good results are to be obtained.

Suggestions are offered, also, for laboratories which prepare their own media and for establishments using desiccated products.

In tropical countries the number of nonselective plates must be increased, because of the greater probability of encountering organisms which require such media *(Sh. dysenteriae, S. paratyphi A, S. sendai,* etc.*)*.

(a) Small Laboratories Using Desiccated Media.—

Inoculate one tube of selenite-F (M-78) *or* tetrathionate broth (M-76). Incubate.

Streak two SS plates (M-67) and one eosin-methylene blue plate (M-71 or M-72). Incubate.

The second day, streak from the selenite-F *or* tetrathionate tube one SS plate (M-67). Incubate this plate for 24 hours.

(b) Small Laboratories Making Their Own Media.—

Inoculate one tube of tetrathionate broth (M-76). Incubate.

Streak two D.E.C. plates (M-68) and one eosin-methylene blue plate (M-71 or M-72). Incubate.

The second day, streak from the tetrathionate broth one D.E.C. plate (M-68). Incubate this plate for 24 hours.

(c) Large Laboratories Using Desiccated Media.—

Inoculate one tube of selenite-F broth (M-76) and one tube of Kauffmann's medium (M-77). Incubate.

Streak one MacConkey plate (M-63) *or* one desoxycholate plate (M-64) and one eosin-methylene blue plate (M-71 or M-72). Incubate.

Streak *either* two SS plates (M-67) *or* one DC (M-65) and one DCLS (M-66) plate. Incubate.

The second day, streak from the selenite-F medium and from Kauffmann's fluid, each, one bismuth sulphite (M-70) and one brilliant green (M-73) plate.

Incubate for 48 hours.

(d) Large Laboratories Making Their Own Media.—

Inoculate one tube of tetrathionate broth (M-76) and one tube of Kauffmann's fluid (M-77). Incubate.

Streak one MacConkey plate (M-63) and one eosin-methylene blue plate (M-71 or M-72). Incubate.

Streak two D.E.C. plates (M-68). Incubate.

The second day, streak from the tetrathionate broth and from the Kauffmann tube to one Hajna-Perry plate (M-69) and one D.E.C. plate (M-68) each.

Incubate the Hajna-Perry plates for 48 hours, the other for 24 hours.

IV. Special Methods

Special methods may be used for the control of carriers or for special surveys. These methods are applicable in closed communities such as military establishments, mental hospitals, etc., and during outbreaks of diseases caused by certain organisms. After the organism has been identified the special methods serve as specialized examinations of contacts and carriers.

(a) Typhoid Bacilli.—

Examine post-cathartic stools in cases without diarrhea. Use stools of patients with diarrhea.

(1) Inoculate one tube of selenite-F M-78 and one tube of tetrathionate broth M-76 with the stool. Incubate for 24 hours at 37° C.

(2) Make an emulsion of the stool in saline.

Place one drop of the emulsion in a sterile Petri dish.

Place 0.5 c.c. of the emulsion in a second sterile Petri dish.

Have ready Hajna-Perry medium M-69 *or* bismuth sulphite agar M-70 melted and cooled to 45° C.

Pour the agar into both Petri dishes and mix well.

Allow to harden and incubate for 48 hours.

(3) Streak from the selenite-F one SS plate M-67 *or* a D.C. plate M-65; from the tetrathionate fluid streak a Hajna-Perry M-69 *or* bismuth sulphite plate M-70 after the enrichment fluids have been incubated. Incubate the plates.

(4) Let enrichment fluids stand at room temperature for 24 hours more and then repeat the streaking (3) and incubating.

(b) Salmonella.—

Examine those cultures from cases and carriers of Salmonellae which form hydrogen sulphide using the same technic as for typhoid bacilli. The Kristensen plate M-73 may be used for streaking from selenite-F or Kauffmann.

Organisms from cases and carriers of Salmonellae which do not form hydrogen sulphide must be examined as follows:

Inoculate one tube of selenite-F M-78 and one tube of tetrathionate M-76 and incubate.

Streak three MacConkey M-63 *or* D.E.C. M-68 plates *or* desoxycholate medium M-64 *or* D.C.L.S. medium M-66. Incubate.

The second and third day, plate from the selenite-F tube and tetrathionate to one plate each of D.E.C. M-68 medium *or* desoxycholate agar M-64. Incubate.

(c) Shigella.—

Collect specimens by means of a Hardy swab and also collect the post-cathartic stools.

Using the Hardy swab, streak either SS agar M-67 *or* D.E.C. plate M-68. Streak sectors on the surface of the medium. Incubate.

Using the stools, streak two plates each of (a) either MacConkey agar M-63 *or* desoxycholate medium M-64; and (b) eosin-methylene blue M-71; (c) D.E.C. medium M-68 *or* SS agar M-67 *or* desoxycholate-citrate plate M-65, and incubate.

If the organisms do not grow on SS agar or on the desoxycholate medium, use the other two plates in triplicate.

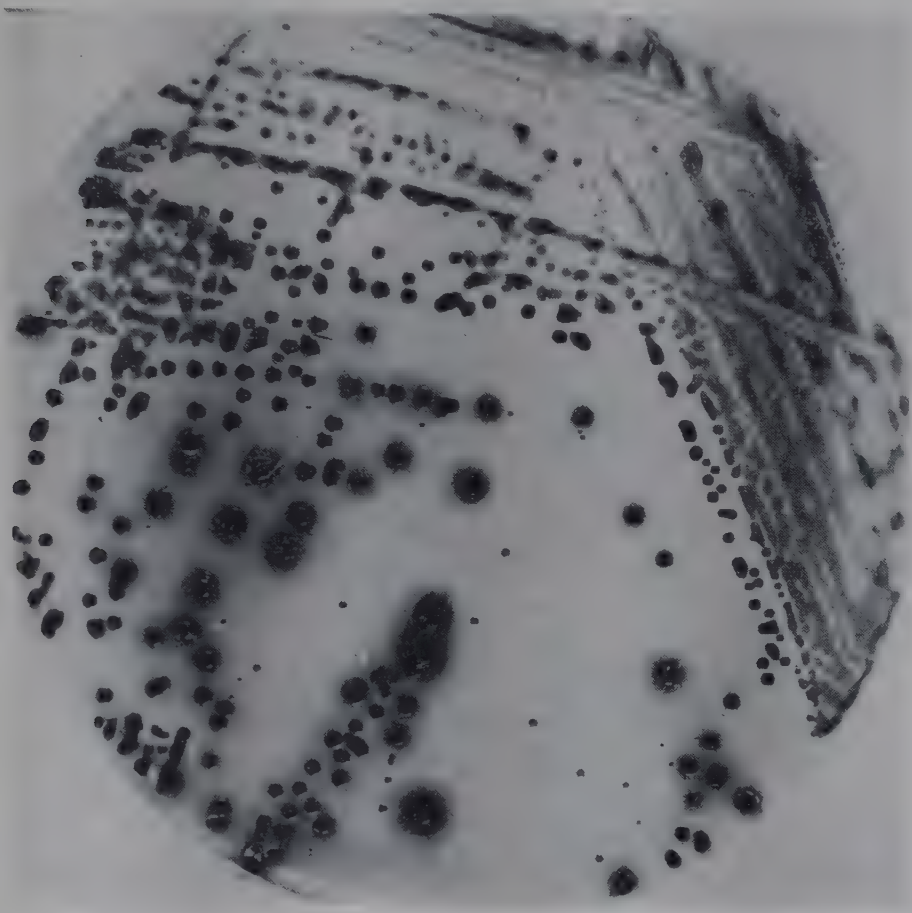

Fig. 392.—Hajna and Perry plate with Salmonellae. (Courtesy Dr. Oscar Felsenfeld.)

V. Technical Suggestions

Use about 1 to 2 grams of inoculum for 10 c.c. of enrichment fluid. Mix the stools well with the fluid. Before plating from the enrichment fluids, shake the liquid.

The above outlines are tentative and substitutions can be made. If substitutions are made, keep in mind the efficacy and selectivity of the different plates and fluids.

Selenite-F, M-78, is preferred by many laboratories to the tetrathionate broth M-76. These enrichments are used only for the propagation of Salmonellae.

The Endo plate and that of Drigalski belong to the past.

Eosin-methylene blue agar M-71, 72 is nonselective and excellent for most fastidious enteric pathogens.

Of the media which inhibit gram-positive organisms by addition of bile, MacConkey's plate M-63 is the least selective, but it gives very large colonies which can be used for direct agglutination tests. Somewhat less effective but much easier to prepare and to streak is the desoxycholate plate M-64 which may be used instead of the MacConkey plate.

The D.E.C. plate of Panja and Ghosh M-68 is the best medium for general use. Unfortunately, some workers consider its preparation a laborious procedure.

SS agar formula M-67 is average in its selective qualities; it is excellent. The desoxycholate-citrate medium M-65 is more selective and more inhibitory for fastidious organisms.

Media which utilize bismuth sulphite are very selective and can be used only for detection of hydrogen sulphide-forming Salmonellae, including the typhoid bacilli. The only disadvantage is the long incubation period (48 hours). The latest modification of Hajna and Perry M-69, and the Difco desiccated product M-70 are widely used.

Kauffmann broth M-77, and Kristensen medium M-73 were devised only for Salmonellae other than *S. typhosa.*

To use nonselective media, inoculate with a small amount of the specimen; selective media require large inocula. Only experience can teach the proper amount of stools to be used for the various plates.

Some media contain sucrose, which permits of the exclusion of a number of Protei and paracolon organisms (D.C.L.S. agar M-66). These plates must be used only when the physician is interested in no organisms other than Salmonellae and Shigellae.

VI. Differentiation of Microorganisms

Those colonies on plates showing reactions of pathogens, black, shiny with metallic halo on bismuth plate; colorless, yellowish, or brownish on others, are transplanted to Kligler agar M-79 or T.S.I. medium M-80. Mickle and Hajna have developed a combination medium which contains three sugars, dextrose, lactose, sucrose, and an iron indicator for the detection of hydrogen sulphide formation. These tubes are inoculated by stabbing deep in the tube, to the bottom, and then streaking the rest of the material on the needle over the slant of the medium. After 24 to 36 hours' incubation, the reactions are read and media for further differentiation are inoculated, according to the organism which is suspected.

Another important characteristic is motility. Motility is determined by inoculating semisolid media M-82, 84, or 88. These media may contain additional carbohydrates, so that fermentation reactions can be obtained along with motility of the organism. Use stab cultures and inoculate by thrusting the wire through the medium. Growth only along the line of stab characterizes nonmotile organisms, like Shigellae, while motile organisms grow into the medium.

All Salmonellae and Shigellae fail to give a positive Voges-Proskauer test R-14. It is advisable, therefore, to determine this reaction.

Mannitol fermentation is used to differentiate subgroups of Shigellae. Protei, with the exception of *Pr. rettgeri,* do not ferment mannitol. The use of this carbohydrate is, therefore, imperative. Use a medium such as M-88. Protei produce urease. They may be excluded, therefore, by testing for urease formation R-15. To do this test, it is necessary to sterilize the urease medium M-90 by filtration.

Many paracolon organisms must be differentiated from Salmonellae by their slow action upon lactose, sucrose, and salicin. Media like M-86, M-87 are recommended. The reaction in medium M-86 is considered positive only when a pink or red color is developed.

Salmonellae do not form indol R-12. This reaction aids in differentiating them from coliforms. This test should, therefore, be made routinely.

Suggestion of Methods for Differentiation of Salmonellae, Shigellae, and Related Organisms

Method 1.—

Discard all Kligler or triple sugar iron agar tubes showing acid slant.

Classify those showing only surface growth and dark discolorization of the slant as *Pseudomonas aeruginosa.*

I. Inoculate from tubes showing alkaline slant, acid and gas in the butt, with or without hydrogen sulphide formation:

(a) one tube of tryptone or trypticase broth M-1
(b) one tube semisolid mannitol M-88
(c) *either* one tube of semisolid sucrose-lactose-salicin M-86 *or* use formula M-87 (optional)

After 24 hours' incubation, make indol test (R-12) in tube (a); read motility and action on mannitol in tube (b).

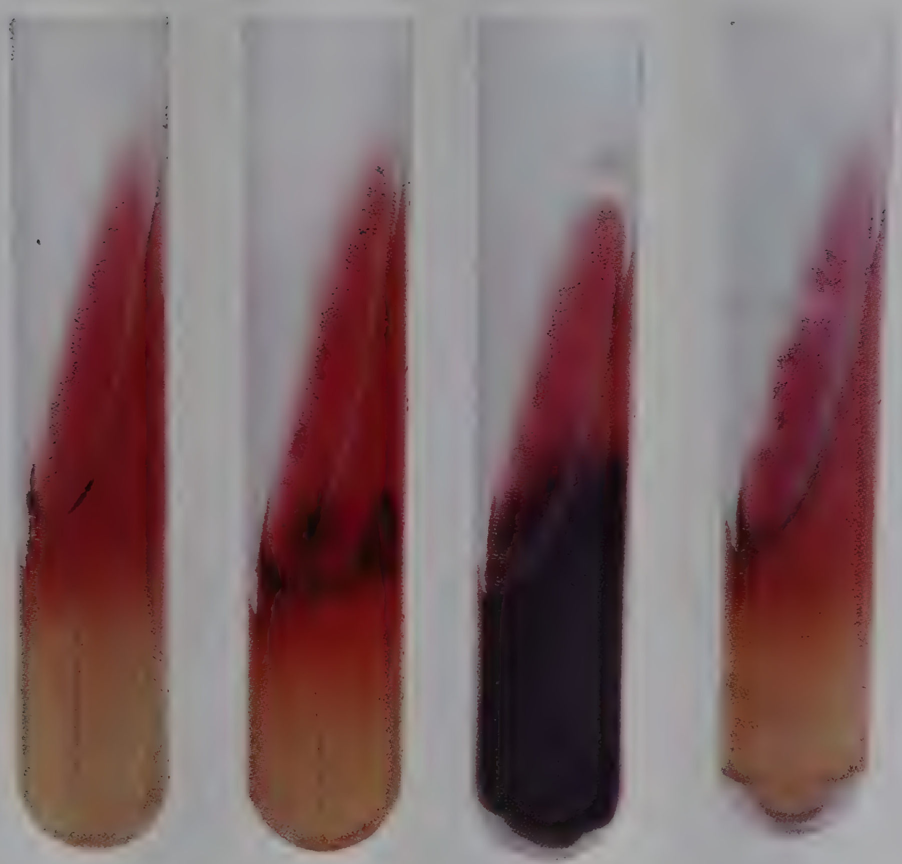

Plate XLII.—Hajna's Triple Sugar Iron Medium. Reaction of Shigella, Salmonella Typhosa, Hydrogen-Sulphide-Forming Salmonella, and Hydrogen-Sulphide-Not-Forming Salmonella.

If indol is negative and acid and gas are formed in mannitol (bubbles of gas in the medium) and no change is noted in medium (c):

Inoculate agar slants for mailing the culture to a Salmonella typing center; or inoculate broth tubes M-1 and agar slants M-16, M-17 for typing.

Reincubate tubes (c) and (d).

Test growth on Kligler or triple sugar iron medium with polyvalent Salmonella sera.

The next day make Voges-Proskauer tests R-14.

If the medium in tube (c) does not show changes, type growth in broth tubes M-1, or mail to a Salmonella center.

II. Inoculate from tubes showing alkaline slant, acid but no gas in butt, and a little hydrogen sulphide formation:

(a) one tube of tryptone or medo-peptone broth M-1
(b) one tube of semisolid mannitol M-88
(c) one tube of semisolid lactose-sucrose-salicin M-86, *or* one tube of semisolid sucrose M-85.

Test the growth on the slant of triple sugar iron agar with (1) polyvalent Salmonella serum (p. 1523); (2) group Salmonella serum D; (3) Salmonella Vi serum; (4) Salmonella "d" serum.

If the serologic reactions are positive, report *typhoid bacillus,* but examine the media for confirmation.

After 24 hours' incubation, make indol test on tube (a). Observe for motility and reaction in tube (b). Keep tube (c) for later reading if agglutination tests are negative.

III. If the growth in Kligler or in triple sugar iron agar shows an alkaline slant, acid butt, and no hydrogen sulphide formation, inoculate:

(a) one tube of tryptone or trypticase broth M-1
(b) one tube of semisolid mannitol M-88
(c) one tube of each: rhamnose broth M-5 and dulcitol broth M-5.
(d) one tube of citrate medium M-93.

Test the growth on the surface of the triple sugar iron agar with (1) polyvalent Salmonella serum, to exclude anaerogenic strains; (2) Salmonella Vi serum, to exclude typhoid bacillus forming little hydrogen sulphide; (3) Shigella sera in the U. S. A. in the order: *Sh. sonnei,* smooth and rough, *Sh. paradysenteriae* Flexner, polyvalent, *Sh. alkalescens-dispar, Sh. boydii.*

If the serologic reactions are positive, test with type sera.

If the serologic reactions are negative, it is better to wait for further testing until the reactions in carbohydrates are read and the growth transferred to at least two agar slants, M-16 or M-17, so as to have sufficient material for further testing.

The second day, make an indol test R-12 on tube (a). Read motility in tube (b). Record the reaction on mannitol and sucrose. Read changes in the carbohydrates in tubes (c). Observe growth on (d).

Make agglutination tests with live and heat-killed growth from the transplants on agar M-16 and M-17.

Keep tubes (c) and (d) until final readings are made.

Table 152 shows the biochemical reactions of the most common Enterobacteriaceae isolated from stools.

The serologic classifications are given in the section describing the various microorganisms.

Method 2.—

Pick suspicious colonies and transfer to a tube of tryptone or trypticase-lactose broth M-5 or M-85. Incubate.

After 4 to 6 hours, if the indicator has not changed color, inoculate from this broth:

(a) one tube of semisolid mannitol M-88
(b) one tube of T.S.I., M-80.

Incubate for 24 hours. Make a Gram stain. Perform indol test in lactose broth (R-12). If gram-negative, and no acidity in lactose broth:

(1) *Organisms nonmotile, acid formed but no gas in (a) and (b):* type with Shigella sera and with Salmonella group IX sera using the growth from the surface of the slant (b). Inoculate carbohydrates for confirmation, as in Method 1.

(2) *Organisms motile, (b) shows acid slant:* discard—coliform.

(3) *Organisms motile, (a) unchanged, (b) alkaline slant, gas in butt:* discard—Proteus.

(4) *Organisms motile, (a) shows only acid but no gas, (b) alkaline slant, acid butt, some H_2S:* test growth on agar slant with Salmonella group D, Vi, and "d" sera.

(5) *Organisms motile, (a) shows acid and gas, (b) alkaline slant, gas in butt, indol test* (R-12) *negative:* keep tube (a) and mail tube (b) to typing center or transfer to further media as in Method 1.

(6) *Only surface growth:* discard—Pseudomonas, Alkaligenes.

Method 3. Alternate Method, Sanders and Okabe.*—

Inoculate lactose broth containing brom cresol purple (M-85).
Four to six hours later, if negative, inoculate from it:
- one tube citrate agar (M-93)
- one tube of semisolid motility agar (M-82)
- one tube of Kligler medium (M-79).

The next morning perform indol test in the lactose broth (R-12).
Set up and perform rapid urease test (R-15B).

This method is especially helpful in regions where Shigellae are frequent, since these organisms do not grow on citrate agar.

Use method 2 only when the technic of isolating and fishing colonies has been well mastered and if sufficient sera are at hand. The Lederle Laboratories, Pearl River, N. Y., produce reliable Shigella species-specific sera, Salmonella group sera, and a few Salmonella single factor sera. These sera must be used in strict adherence to the instructions of the manufacturers, if good results are to be achieved.

Method 4. Broh-Kahn procedure† for routine stool cultures is as follows:

All specimens, either anal swabs or actual stools, should be streaked to plates of SS agar M-67 and brilliant green agar M-72, and, in addition, inoculated into tubes of tetrathionate enrichment broth M-76, from which a second brilliant green plate M-73 is streaked the next day. Typical colonies should be studied both by inoculation into tubes of Kligler's agar M-79 and by agglutination with polyvalent sera. Owing to the selectivity of Kauffmann brilliant green agar, cultures which show typical reactions in either the polyvalent sera or the double sugar agar may safely be reported as possible Salmonella and should then be forwarded to a Salmonella Center for confirmation and species identification.

The SS agar plates should be examined for growth of dysentery strains and the presence of the latter demonstrated by conventional methods.

Method 5. Braun's Two-Tube Method.‡—

Melt tubes of Braun's medium *B* (M-81) and keep them at 45° C. in a water bath.

Pick a colony and transfer part of it to one Braun tube *A* (M-81), inoculating slant and stabbing it, and part to one tube of melted medium *B* (M-81), preparing a shake culture in the latter.

Insert indol reaction paper R-12d between plug and neck of tube *A*.

Insert hydrogen sulphide indicator paper R-9 between plug and neck of tube *B*.

Incubate for 24 hours at 37° C. and read results.

All Salmonellae and Shigellae produce a blue slant and a green butt in tube *A*.

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*U.S. Armed Forces M. J. 4: 1053, 1953.

†Mil. Surg. 99: 770, 1946.

‡Istanbul Seririyati 21: 3, 1939.

Mannitol fermentation is indicated by a yellow color in tube *B*, which will show gas formation, too.

Alcaligenes faecalis does not grow in the depth of medium *B*.

Method 6. Micromethods.—

For rapid identification of Shigellae and Salmonellae, medium M-95 may be used, in conjunction with a preliminary identification medium such as M-85, M-87, M-90, or M-91. Such preliminary or primary medium will produce enough growth after 4 to 6 hours' incubation that further media, especially M-89, can be inoculated from it.

The initial growth in M-85 can be used for rapid agglutination tests.

Attention must be called to the fact that there are many pitfalls in using rapid methods. They should not be used except by those whose technic is flawless, and in emergencies, such as in outbreaks, where a rapid diagnosis is most essential.

An infinite number of combinations and variations of the media and methods can be set up. The two most useful are:

(a) *Pick each suspicious colony and transfer simultaneously to:

One tube of lactose broth with indicator M-85.

One tube of semisolid mannitol with yeast extract M-89.

Incubate for 4 to 6 hours and observe reactions. If the indicator in the lactose broth has changed, discard.

If the indicator in the lactose broth has not changed, transfer part of the lactose broth to an empty, sterile, small test tube.

Observe the mannitol tube, preferably with the aid of a dissecting microscope.

If mannitol negative, no motility, no hydrogen sulphide:

Test growth in broth which was transferred to the empty tubes with Shigella group A, B, C, and D, and Salmonella polyvalent sera. (Some typhoid bacilli will give this reaction, too.)

If mannitol tube shows acid production around the stab but no gas, no hydrogen sulphide production, no motility:

Test with Shigella group B, C, and D, and Salmonella polyvalent sera. (Some typhoid bacilli will give this reaction.)

If mannitol tube shows acid, no gas, no hydrogen sulphide or some hydrogen sulphide production, some motility:

Test with Salmonella sera, especially for *S. typhosa.*

If mannitol tube shows acid and gas production, hydrogen sulphide (most frequently) produced, motility present:

Test with Salmonella polyvalent serum.

There is seldom enough growth in the lactose broth to complete the typing. It is essential to inoculate only small amounts of broth with the colony, otherwise after four hours there will not be a dense enough suspension available.

The usual method of agglutination is that with sera diluted 1:5, using the slide test. It is possible to perform tube agglutinations, using 0.2 c.c. of the growth in lactose broth and 0.2 c.c. of the proper serum dilution. This is, however, difficult to read.

The mannitol tube serves as a source for further inocula. An indol test may be made the next day with the lactose broth.

(b) Pick colonies and transfer to urea broth M-90 or M-91.

Incubate for 4 to 6 hours; then, if negative, inoculate M.R.-V.P. medium M-92 for rapid Voges-Proskauer test (R-14d), and broth for rapid indol test (R-12).

Prepare sterile Petri dishes and, after properly marking sectors, put into them drops of agar M-95 containing:

lactose
sucrose
mannitol
motility medium with hydrogen sulphide indicator.

Inoculate the drops by stabbing.

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*Goldner, H.: Am. J. Digest. Dis. 21: 198, 1954.

The preferred technic is to use drops with one carbohydrate in the same plate.

Incubate the Petri dishes for 2, 4, and 6 hours.

Observe under low power, or with a lens, for motility and fermentation along the stabs.

Re-read the urea medium.

Read the Voges-Proskauer and indol tests.

Cocci

Not many conclusions can be drawn from the presence or absence of certain cocci in the feces. This question is often raised when staphylococcus food poisoning outbreaks are investigated. This type of food poisoning is caused by the toxin of the organisms formed during their growth in food; thus the presence or absence of hemolytic staphylococci in the stools has little significance.

If enterococci are to be isolated, streak the stools on two sodium azide blood plates M-21 and test the cocci as follows:

Inoculate a tube of trypticase soy broth M-3.

After incubating 24 hours, heat the tube for 30 minutes in a water bath at 60° C. The enterococci survive.

Inoculate from the heated broth

(a) one tube of Chilton and Fulton medium M-87. Observe for blackening.

(b) Add 0.5 c.c. of the broth in which the cocci were grown to 0.5 c.c. of a 5% suspension of washed sheep red blood cells. Incubate for 2 hours in a water bath at 37° C.

This divides the enterococci into two groups, hemolytic and nonhemolytic.

Attempt typing with Lancefield D serum, if hemolytic.

Cholera

Tests for the presence of cholera vibrios are made only in regions where cholera is present or when suspicion arises as to a possible diagnosis of cholera.

Mix 1 to 2 gm. of the stools in tubes containing alkaline sea-salt medium M-90 and alkaline peptone water M-1. The former is a good preservative and may serve as a preservative for mailing stools or water.

Streak the stools directly on two D.E.C. plates M-68 and two Aronson plates M-74.

Incubate for 24 hours. Pick colonies from the plates.

Streak out colonies from the sea-salt medium to a Wilson and Reilly plate M-75.

Incubate, then pick colonies as above.

Transfer suspicious colonies to alkaline tryptone broth M-1.

Incubate for 6 to 8 hours and inoculate from the tryptone water:

(a) one tube of agar slant M-16 or M-17
(b) one tube of sucrose broth M-5;
    one tube of arabinose broth M-5
(c) one tube of nitrate broth M-8
(d) one tube of Voges-Proskauer medium M-92.

If possible, test the colonies with polyvalent cholera serum directly, using the spot agglutination method.

The second day, make indol test (R-12) in tube of tryptone broth M-1.

Observe if sucrose was fermented and arabinose remained unchanged.

Make the cholera red test (R-16) in tube (c).

Carry out agglutination tests with the growth on agar slants (a).

Observe tubes (b) for 5 more days.

Make the Voges-Proskauer reaction on the third day with the growth in tube (d) (R-14).

Test for hemolysis, using 5% suspension of washed goat cells.

Tuberculosis

(Petroff's Method)

Collecting the Specimen.

The morning stool is collected in a wide-mouth sterile fruit jar. See collection of specimen in Chapter VIII.

Concentration of the Specimen.

Add to the stool 2 volumes of distilled water.

Stir thoroughly and filter through gauze to remove the coarse particles.

Add enough sodium chloride crystals to make a saturated solution.

Mix thoroughly and allow to stand at room temperature for several hours. The bacteria float on the surface, and may be collected in the scum.

Collect the scum by means of a sterile spoon and place in a sterile wide-mouth bottle.

Add 2 volumes of N/1 sodium hydroxide. Shake thoroughly.

Place in the incubator at 37° C. for one to two hours.

Centrifuge at high speed.

Decant and discard the supernatant fluid.

Add to the sediment 3 or 4 drops of N/1 hydrochloric acid.

Divide the sediment into three portions.

Make 2 smears with one portion. Stain with Ziehl-Neelsen.

Examine for tubercle bacilli.

Spread one-third of the sediment on media M-58, M-58a, M-58b, or M-59, M-60, and incubate, making allowance for exchange of gases by punching a hole through the corks. Incubate egg media six to eight weeks. Growth occurs more rapidly on Petrik medium.

The other third should be injected into a guinea pig (page 1647), since the finding of acid-fast bacilli in the feces does not necessarily mean the presence of tubercle bacilli. If the guinea pig dies of tuberculosis, or shows lesions of tuberculosis, report: Tubercle bacilli present in specimen.

Animal inoculation is the method of choice.

Brucellosis

Examination for brucellosis by examining the feces is usually a futile attempt.

Streak the stool on tryptose agar plates M-16 or liver infusion agar M-26 and incubate in a carbon-dioxide atmosphere.

Fish suspicious colonies and transplant to liver infusion agar with dyes M-26^a for differentiation.

Gonococci

Cleanse anal opening.

Introduce anoscope.

Cleanse lesions with saline.

Take material as from the urethra and proceed according to instructions for urethral, prostatic, and cervical material.

URETHRAL, PROSTATIC, AND CERVICAL MATERIAL

Smears of urethral pus are very important for bacteriologic diagnosis of the diseases of the genitourinary tract, but they should be backed with proper bacteriologic culture methods.

In the male, the smears of urethral pus must be taken in the following manner: the glans penis is cleaned with alcohol, especially around the meatus. A platinum loop is then very carefully inserted into the urethra and a drop of pus is picked up and inoculated upon medium in a Petri dish. In cases where it is necessary to examine material from the deep urethra, the use of a long sterile probe through an endoscope with electric illumination is necessary. For the examination of prostatic and seminal vesicle material for gonococci, the glans should be disinfected with alcohol and, with the patient bent over, the prostate and vesicles are massaged, allowing the material expressed from these organs to run into a sterile Petri dish. From this dish, inoculation is then made on the gonococcus medium.

An alternative method is to press on the urethra, massage the prostate, and then release the pressure on the urethra. The outflowing material is collected into a tube or Petri dish.

In the female, inoculations are made from the urethra and from the vagina. In children, a female glass catheter is introduced into the vagina and rotated. When sufficient material is collected, the vagina may be washed with a few c.c. of tepid saline.

Cervical material is collected through a speculum. The cervical plug, if present, is cleaned out and the material is collected from the introit.

Make cultures on Gradwohl plate M-37 or on chocolate agar M-31, M-32, M-33, M-36. Incubate the plates in a carbon-dioxide atmosphere and examine after 48 hours with the aid of the oxidase reaction. The colonies on Gradwohl plate have the following characteristics:

1. **Gonococcus (Neisseria Gonorrhoeae).**—Twenty-four-hour colonies are distinct, flat, gray, larger than streptococcus colonies, but usually smaller than staphylococcus colonies, although they vary greatly in size. Granules, while fine, are very dense. Occasionally twenty-four-hour colonies show one or two radial striations, or a concentric ring. If held up to the light, so that the light shines through them, they exhibit a pearly opalescence.

They are almost transparent. The iris diaphragm of the microscope must be nearly closed to obtain a clear picture of the structure.

Margins of the twenty-four-hour colony are fairly regular. There is no central structure.

Colonies show changes in forty-eight hours. As they become older, autolysis of the organisms takes place, thus changing the size and structures of the colonies. The edges become undulated, the center flatter than the edges, and radial striations become marked, although they are visible only when the light is cut down. Occasionally there will appear in the center of the colony a crumblike structure. This structure does not add opacity to the colony, which remains almost transparent throughout the course of its growth. Smears from forty-eight-hour colonies almost invariably show marked autolysis.

Seventy-two-hour and ninety-six-hour colonies show changes in size; that is, the colony enlarges. The edges tend to become lobate, the striations marked, and many of the crumblike structures may appear. Smears from old colonies will show that the majority of the organisms have undergone autolysis. The medium remains purple. (See Figs. 393*A*, 393*B*, and 394.)

2. **Neisseria Catarrhalis.**—The 24-hour colony of the *Neisseria catarrhalis* is almost identical with that of the gonococcus, although there are never any striations, and the granules seem less dense. The older colonies of the catarrhalis do not show much change, although they assume a shriveled appearance microscopically. The colonies may have concentric rings. Like the gonococcus, the colonies are gray. They do not react with the sugar; therefore, they do not turn the medium yellow. No autolysis is noted.

3. **Gram-Negative Streptococci.**—Colonies, macroscopically, are distinct, elevated, shiny, pinpoint. They are surrounded by a yellow zone of fermentation. This color is sometimes not visible within twenty-four hours, although, if the plates are held up to the light, the evidence of fermentation may usually be noted. They have a deep center, and a lacelike periphery, the edge being slightly undulating.

Smears from the colonies show typical chain cocci which stain gram-negative.

They should never be confused with gonococcus colonies, since they are uniform in size, shiny, and have a yellow zone around them.

4. **Colon Bacillus (Escherichia Coli).**—The identification of this organism is not important in the diagnosis of gonorrhea, although its differentiation from the gonococcus is important.

Colonies are about the size of a staphylococcus colony. They are flat and dull to shiny, and usually ferment the levulose of the medium, giving a yellow zone around them. If this fermentation is delayed, they can be distinguished from the gonococcus colonies by the fact that they are moist, whereas the gonococcus colonies are fairly dry.

Microscopically, they are evenly granular, semitranslucent, with a rather uneven margin. Smears show typical gram-negative bacilli of the morphology of the colon bacillus.

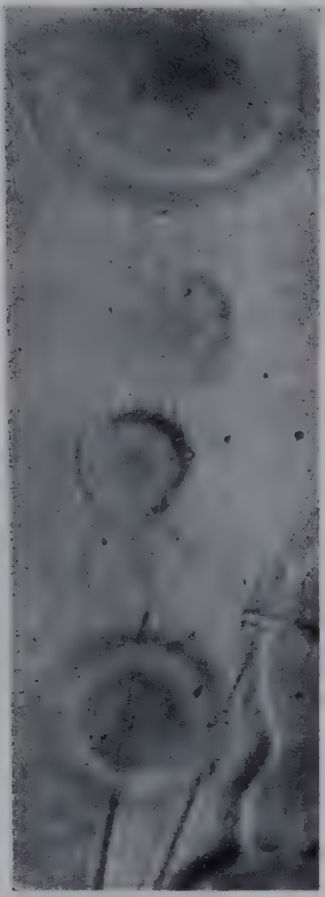

Fig. 393*A*.

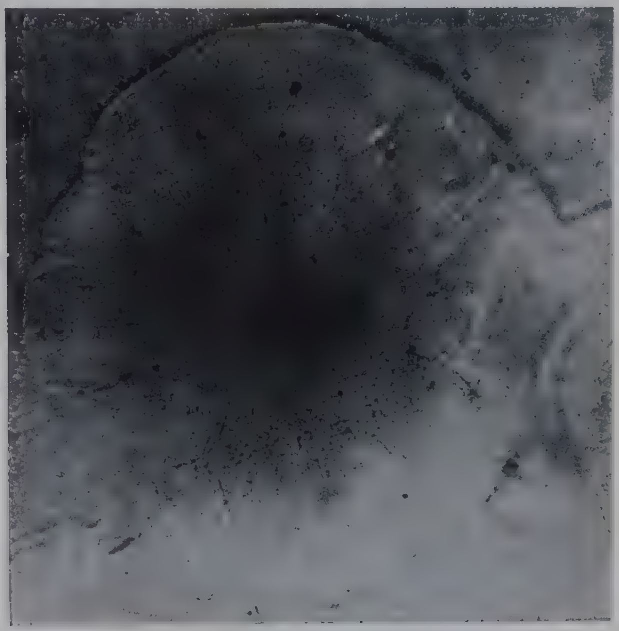

Fig. 393*B*.

Fig. 393*A*.—Twenty-four-hour colonies of *Neisseria gonorrhoeae* on Gradwohl gonococcus medium growing along the streak made by the platinum wire. Note the concentric rings.

Fig. 393*B*.—Forty-eight-hour colonies of *Neisseria gonorrhoeae* on Gradwohl agar. Note the radial striations of the large colony and the concentric rings of the small colony.

5. **Friedländer Bacillus (Klebsiella Pneumoniae).**—These colonies may be differentiated from the gonococcus colonies by the fact that they usually show a white pigment. They are slimy, and semitransparent. Smears show typical gram-negative bacilli.

6, 7, 8. **Diphtheroids.**

Three types of diphtheroids have been noted.

6. **Pinpoint colonies,** distinct, and possessing a deep center; they are never confused with gonococcus colonies. They show this deep center macroscopically as well as microscopically. Edges are even. Emulsification of the colony is quite difficult, since the organisms tend to stick together. Smears show typical diphtheroids, usually stained gram-variable.

7. **White pigmented colonies,** slimy, small, sometimes fermenting the levulose, sometimes not reacting with the sugar. Morphologically, they are club-shaped diphtheroids with granules or bars of metachromatic substance.

8. Colonies are **flat, transparent, distinct, small,** and do not ferment the levulose. They, therefore, do not show a yellow zone around them. A beginner may mistake them for gonococcus colonies, if the macroscopic appearance alone is studied. Microscopically, they show fine, regular granules at the periphery; the central portion is composed of rather coarse, large, round granules that give the appearance of a collection of crude projections from the surface. There is no distinct central structure, and no radial striation. They are less densely granulated than colonies of gonococci, and more translucent. Smears show typical diphtheroids.

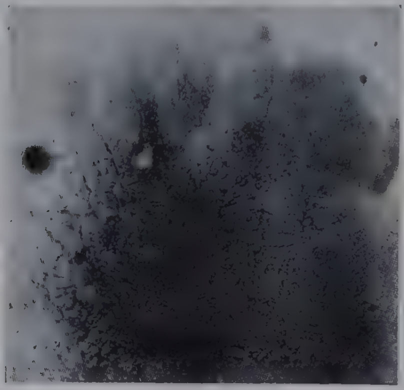

Fig. 394.

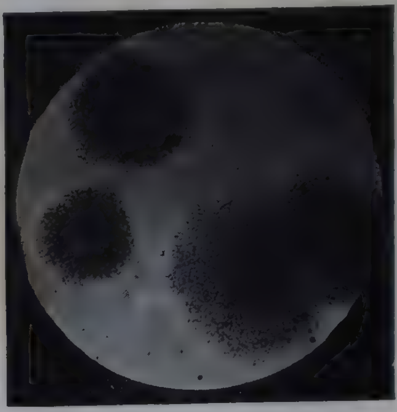

Fig. 395.

Fig. 394.—Ninety-six-hour colony of *Neisseria gonorrhoeae* on Gradwohl agar showing deep lobulation, radial striations, and coarse "crumbs." Only a portion of the entire colony is shown.

Fig. 395.—Colony of *Streptococcus mitis* on Gradwohl agar.

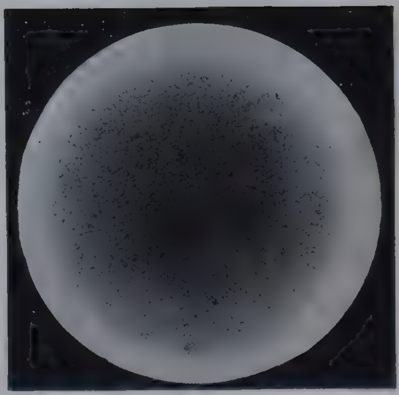

Fig. 396.

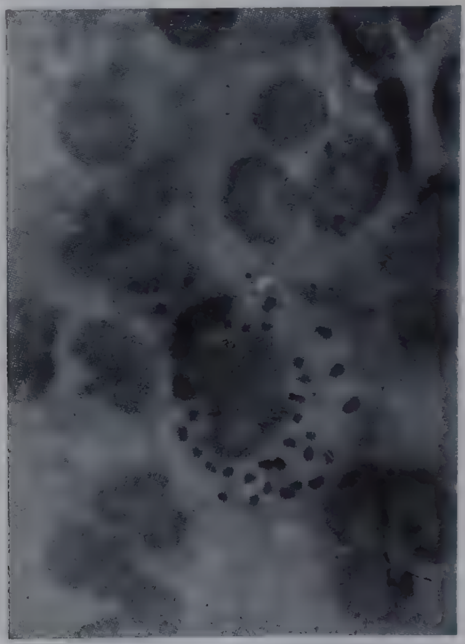

Fig. 397.

Fig. 396.—Colony of a diphtheroid on Gradwohl agar.

Fig. 397.—Gonococci in a pus cell (×1,000).

9. **Staphylococci.**—Colonies are characteristic; that is, they are circular, shiny, opaque, with unbroken margin, and produce pigments. They ferment the levulose, showing a yellow zone around the colony. Stained preparations show typical staphylococcus formation; that is, spherical organisms in grape-like formation, taking a design-like appearance. If fermentation of the levulose is complete, they may stain gram-negative instead of gram-positive.

10. **Sarcinae.**—These colonies resemble the colonies of staphylococci. Smears show spherical organisms in bundle formation. They may stain either gram-positive or gram-negative.

11. **Air Contaminants.**—Air contaminants can always be recognized by their size and the location on the plate. They usually are not on the streak made by the platinum wire.

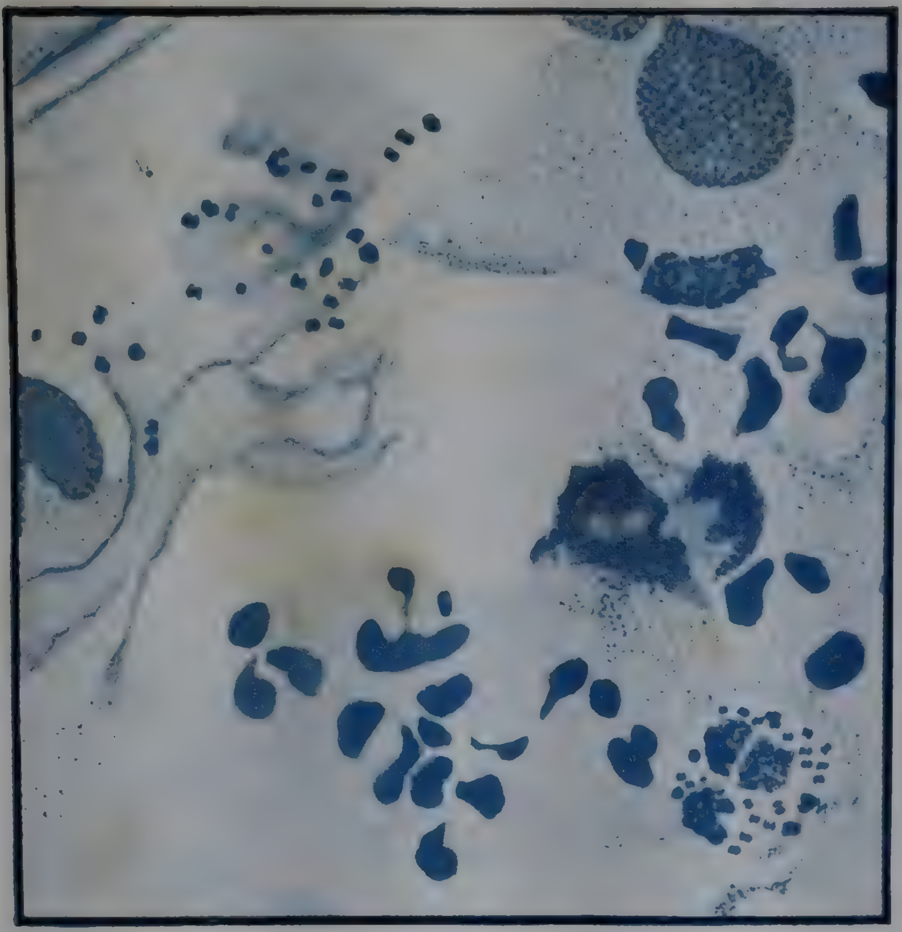

Plate XLIII.—Gonococci in Pus, Methylene Blue Stain.

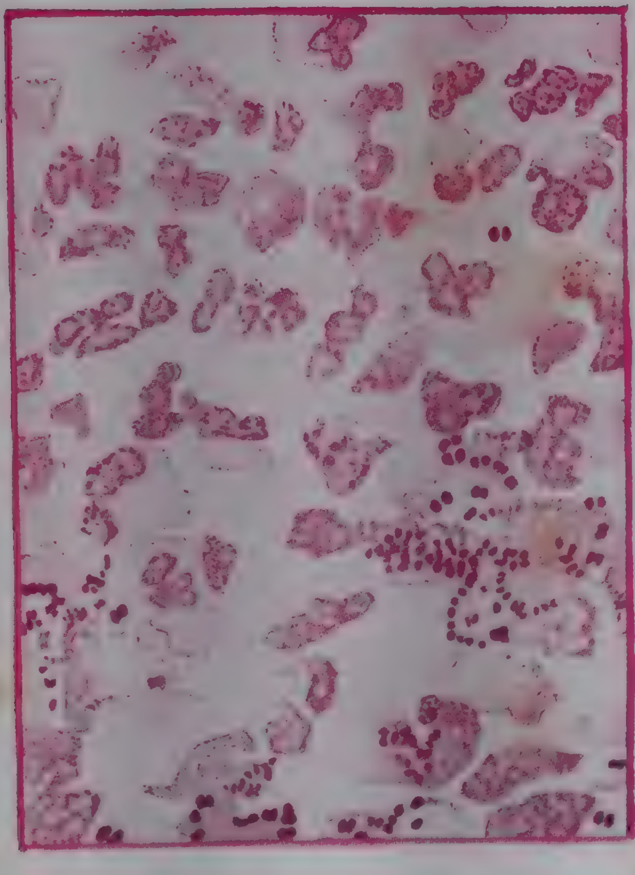

Plate XLIV.—Gonococci in Pus from Urine Sediment, Gram Stain.

If it is impossible to culture the material immediately, it should be kept in a cold place, to prevent overgrowth of contaminating organisms.

Public health laboratories have sought a method of keeping cultures or inocula of gonococci alive in transit: most of the cultures submitted by physicians to such laboratories are necessarily sent by mail. It is well known that in most cases, unless the material inoculated with the suspected gonococci reaches a laboratory very quickly, no growth will be obtained and the whole purpose of gonococcus culture is without results. An ideal medium for such purpose would be one that would maintain the viability of the gonococcus and inhibit the growth of other bacteria at the temperatures to which the medium might be exposed en route.

Alternative media for preservation and mailing were suggested by Hirschberg (M-34) and Sanders (M-35).[1]

Place 3 c.c. of the media with sterile precautions in a suitable vial (approximately 120 by 22 mm.)[2] containing a plastic screw cap (phenol Formalin-free) with a well which holds the cotton swab applicator. The applicator should be long enough to reach the bottom of the vial. In this way the swab is practically imbedded in the medium. Add carbon-dioxide gas with sterile precautions after the agar has solidified. Securely tighten the caps. Test the vials for sterility, and store at room temperature until used, preferably within 1 week. The vials should be kept upright at the time of use in order to avoid excessive loss of CO_2.

These media prevent the growth of many contaminants which ordinarily would overgrow and prevent the isolation of the gonococcus.

Smears of Pus or Urine for Gonococci

(From Discharge Pus, etc., or Urinary Sediment.)

If the pus is obtained from urine, the urine must be centrifuged at high speed for about fifteen minutes, and the sediment smeared on glass slides.

Fix the slides in the flame.

Stain with Gram stain.

When decolorizing, keep the slide in the alcohol until the pus is colorless.

Examine for bean-shaped diplococci, intracellular or extracellular.

Report.—Gram-negative diplococci **resembling** gonococci. Report pus cells, epithelial cells, and other bacteria. No definite identification of the organisms can be made by the smear method. Culture methods are the only means of absolute identification of gonococci. If negative, report no gonococci found.

Dried Pus for Gonococci

Place the material to be examined in a Petri dish.

Soak off the dried pus in a small amount of sterile physiologic saline.

Plate out on several plates of special media M-31, 32, 33, 36, or 37, and incubate, lid on top, for twenty-four to forty-eight hours.

Identify the colonies microscopically, and confirm their presence by stained smears from the colonies.

Report: Gonococci found, or no gonococci found.

For other organisms causing inflammation and pus formation, proceed as for the examination of fistulas.

Special methods are used for the investigation of infections with *H. ducreyi.*

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[1] J. Ven. Dis. Inform. 28: 6, 1947.

[2] Glassco No. 7350.

Diagnosis of Hemophilus Ducreyi by Smears

Kornblith* in a series of 175 cases reported that the smear was the best single criterion of diagnosis, positive identification being possible in 88.57 per cent of their cases. Greenwald** uses the Gram stain. In his hands the smear was positive in 65 per cent of his cases.

Some workers prefer Wright stain or methyl green pyronin.

Cultivation of the Hemophilus Ducreyi (Bacillus of Soft Chancre)

Method of Sanderson and Greenblatt

Sanderson and Greenblatt advocate the following method for the cultivation of *Hemophilus ducreyi*[1, 2, 3]: Soft beef infusion agar (with only 0.5% agar) M-15 is put up in aluminum screw cap vials of 10 c.c. capacity, in 5 c.c. amounts and sterilized. When ready for use, 1 c.c. of human or rabbit defibrinated blood is added per vial. Bubo pus is aspirated under strictly aseptic conditions to prevent admixture of skin contaminants, and not more than 0.2 c.c. inoculum to a vial. The culture is incubated at 37° C. Growth in filaments or long chains of gram-negative bacill usually occurs in 48 hours, although cultures should not be discarded as negative within a week. Sometimes in initial cultures the bacilli are to be found in masses of tangled clumps, and it may take careful search to locate them. Once grown, the cultures must be transferred every few days to retain viability, until the organisms have become adjusted to the new environment. They may be then subcultured at weekly to ten-day intervals.

Little success is had in obtaining pure cultures from ulcers or buboes that have become infected with secondary invaders owing to the more rapid proliferation of these organisms. As a general rule, *H. ducreyi* grows poorly or not at all on the surface of culture media. Hence, the usual plating methods are of little value in the isolation of this bacillus. Unruptured buboes offer the best source for obtaining pure cultures.

For the detection of *Treponema pallidum,* dark-field examination or tissue biopsy may be necessary.

Dark-field Examination for Treponema Pallidum (Spirocheta Pallida)

The organism which causes syphilis is the *Treponema pallidum,* which was described by Schaudinn and Hoffmann in 1905. They can be identified readily by their movement in spirals with dark-field examination. For details in preparing specimen see Chapter I, page 11. This organism is a delicate, thin spirocheta ranging from 6 to 15 microns long and from 0.25 to 0.3 microns wide. The spiral amplitude is 1.0 micron, regular. The spiral depth is 0.5 to 1.0 micron and very constant. Waves, one or more, slight, undulating curves may be present. Progress is due to the rotary motions with undulations with some expansion and contraction of the coils. There is an absence of the chambered structure. If a membrane is present, it must be flexible. Flagella are absent. A highly motile end portion is absent.

In the preparation of the specimen for the diagnosis of syphilis with the dark-field examination, one must be careful to avoid confusion with other spirochetes. For instance, preparations from the mouth for dark-field exami-

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*J. A. M. A. **117**: 2150, 1941.

J. A. M. A. **121: 9, 1943.

[1] South. M. J. **30**: 147, 1937.

[2] Am. J. Clin. Path. **7**: 193, 1937.

[3] Arch. Dermat. & Syph. **36**: 486, 1937.

nations for *Treponema pallidum* must be made very carefully and one must bear in mind the possibility of differentiation of the species from the *Treponema microdentium,* which resembles the *Treponema pallidum* very closely. In general, spirochetes other than the syphilitic organism have slower motion, greater width, and longer spirals. This is particularly true of the *Borrelia refringens,* which is found upon the genitals. There should be no difficulty in making a diagnosis of *Treponema pallidum* by dark-field methods from preparations from extragenital chancres on the lip, face, hands, and from genital chancres, but in respect to lesions within the mouth, one must be very careful in not confusing the *Tr. pallidum* with the *Tr. microdentium.*

Diagnosis of Congenital Syphilis by Dark-Field Methods.—The diagnosis of congenital syphilis is very important. Demonstration of spirochetes by dark-field examination of fresh preparations from the wall of the umbilical vein has been recommended by Norman R. Ingraham, Jr.[1]

He presents results of dark-field examination from scrapings from the umbilical vein in diagnosis of congenital syphilis. Spirochetes have been found in the blood stream, in the nasal secretion, in conjunctival secretion, syphilitic cutaneous lesions, the urine, and in the meconium of syphilitic infants. Spirochetes have been described as present in the umbilical cord by a number of European workers. Huebschmann and Simmonds in 1906 found spirochetes in sections of umbilical cord stained by the Levaditi method. This was confirmed by others. Emmons found spirochetes in silver stained sections of the umbilical vein in only one of fifteen syphilitic infants. Manouélian found spirochetes in congenital syphilis in cases of periphlebitis of the umbilical vein.

Demonstration of spirochetes by dark-field examination of fresh preparations made from scrapings from the wall of the umbilical vein was mentioned by Gräfenberg as early as 1908. It was rediscovered by Vulovic in 1923 and has been employed by others.

The **method of Vulovic** used by Ingraham was as follows:

> Immediately after delivery, a portion of the umbilical cord, about 3 inches long, is cut from the distal end, that is, the end nearer the child, and placed in a sterile moist bottle without preservative. Examination must be made as soon as possible. Examination is made by dividing the specimen in half and looking for the two arteries and the single vein. Pass through the vein a small blunt instrument, like a probe, and cut longitudinally to expose the lumen. Blood adhering to the intima of the vessel is scraped away with a knife. Fresh scrapings are made from the wall of the vessel, placed in physiologic solution of sodium chloride, macerated, placed on a slide and examined in the dark-field microscope.

Ingraham found this method useful in nineteen of eighty-seven cases, or 21.8 per cent.

Diagnosis of Syphilis by Examination of Biopsy Tissue.—(See Method of Krajian, page 2138.)

URINE

1. Urine for bacteriologic examination should always be collected by catheterization under very proper sterile and aseptic technic, into a sterile container.
2. The examination of urine for Spirochaetales, helminthic eggs, and Trichomonas is described in Chapter XII.

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[1] J. A. M. A. **105**: 560, 1935

3. See pages 1521 ff. and 1632 ff. for examination for typhoid and other Salmonellae.

Transfer urine, with all sterile precautions, to two sterile corked test tubes.

Centrifuge at high speed for one-half hour.

Remove the supernatant fluid with a sterile dropper with rubber bulb. Always place the supernatant fluid from any material which may contain bacteria into a container which can be placed in a sterilizer and destroyed.

The sediment is used for inoculation.

If no definite information is given as to the probable type of organism which may be found, a complete bacteriologic examination must be made for

1. Streptococci
2. Staphylococci
3. Pneumococci
4. Gonococci
5. Coliform organism and Salmonellae
6. *Pseudomonas aeruginosa*
7. Tubercle bacilli, etc.

4. With the sediment inoculate
   - (a) one blood agar plate M-28, M-29
   - (b) one eosin-methylene blue plate M-71, M-72.

If gram-positive cocci and gram-negative rods are seen in the smears, inoculate as well
   - (a) one sodium azide blood plate M-21, 22, or alcohol plate M-23.
   - (b) proceed with the examination of colonies as described under "Pus."

Inoculate also one gonococcus plate (M-35 to M-37) and incubate in carbon-dioxide atmosphere. Proceed as for gonococcus examination of urethral material.

For the isolation of Salmonellae, inoculate one tube of selenite-F M-78 with the urine. Use about 2 c.c. of the urine for each tube.

Incubate for 24 and 48 hours. Streak the growth from the tubes on SS plate M-67 *or* desoxycholate-citrate M-65 plates and on bismuth sulphite M-70 plates. Proper modifications or media made by the laboratory (not from desiccated products) may be substituted.

Proceed according to the directions under "Stool."

Examination of Urine for Tubercle Bacilli

The finding of an acid-fast bacillus in the urine is not necessarily evidence of tuberculosis. Because of the possibility of the presence of the smegma bacillus (*Mycobacterium lacticola)* in the genitourinary tract, it is necessary either to cultivate the organism, or better, to inoculate a guinea pig.

Collecting the Specimen.[1]—

Prepare 1 or 2 liter bottles by washing well in alkali, followed by washing in potassium dichromate-sulphuric acid cleaning solution. Rinse thoroughly in tap water, then in distilled water, cork well, and sterilize in the dry heat sterilizer at 170° C. for two hours. Wrap a glass funnel in a towel, and sterilize in the same manner.

Instruct the patient to collect the morning specimen, and the remainder of the specimen for the twenty-four hours until the following morning. The patient should also be instructed not to touch the inside of the bottle with the fingers, and to avoid as far as possible contaminating the specimen in any manner whatsoever.

No preservative should be used, although a small crystal of thymol or camphor may be added if the specimen is to be sent some distance.

(a) Direct Examination:

Allow the sediment to collect. Select especially the shreds of fibrin and pus. A smear is made, and stained with the Ziehl-Neelsen stain after fixation. It is well to make the smears in a loopful of serum or egg albumen to avoid the possibility of washing the smears off the slide in the process of staining.

Tubercle bacilli are characteristic, although no definite diagnosis may be made by this method.

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[1] Baldwin, Petroff, and Gardner: Tuberculosis; Bacteriology, Pathology, and Laboratory Diagnosis, Lea & Febiger, 1927.

Report: Acid-fast bacilli resembling tubercle bacilli.

(b) **Sedimentation Method** (Petroff):

Use the twenty-four-hour specimen collected in the manner described above.

If not acid, acidify by adding a few drops of 30% acetic acid.

To every 1000 c.c. of the acidified urine add 2 c.c. of 5% solution of tannic acid. Shake thoroughly.

Place in the ice box for twenty-four hours. A heavy precipitate will collect at the bottom of the bottle.

Decant and discard the supernatant fluid.

Centrifuge the sediment at high speed for ten minutes.

Decant and discard the supernatant fluid.

To the sediment add 1 c.c. N/1 sodium hydroxide to dissolve it.

Warm in a water-bath at 37° C. for thirty minutes.

Dilute with 3 volumes of sterilized water.

Mix thoroughly and centrifuge at high speed.

Decant and discard the supernatant fluid.

Spread the sediment on slides, making the smear in either serum or egg white. Fix in the flame, and stain with the Ziehl-Neelsen stain.

Tubercle bacilli are characteristic, but cannot be definitely identified by the smear when found in urine.

Report: Acid-fast bacilli resembling tubercle bacilli.

(c) **Animal Inoculation** (Petroff):

This is the method of choice for the identification of tubercle bacilli in urine.

Prepare the sediment from a twenty-four-hour specimen in the manner described above (Petroff).

Inject the entire sediment subcutaneously into the skin fold of the right inguinal region of 2 guinea pigs.

Tag the pigs, and record on the laboratory chart, giving the date of inoculation, the number and weight of the pigs, and the identification of the specimen.

If pigs die within a few days, contaminating organisms have probably caused the death.

Five weeks, or longer, after the injection, a tuberculin skin test is made on the animals.

If the animals do not react, they should be kept a few weeks longer before an autopsy is performed.

If, after two months, the skin test is still negative, one of the pigs is sacrificed with chloroform, and autopsied.

The other pig is retained for several months.

If there were any tubercle bacilli in the specimen, the pigs will show lesions of tuberculosis on the spleen, the kidneys, the liver, or other abdominal organs.

(d) **Isolation and Cultivation of Tubercle Bacilli from Urine** (Petroff):

Prepare a sediment from a twenty-four-hour specimen in the manner described above (Sedimentation Method, Petroff).

To the sediment, add 5 volumes of N/1 sodium hydroxide.

Shake thoroughly.

Incubate in a water-bath at 37° C. for thirty minutes.

Centrifuge for five minutes at 2000 revolutions per minute.

Decant and discard the supernatant fluid.

To the sediment, add 2 drops of N/1 hydrochloric acid.

Distribute the entire sediment by means of a capillary pipette over the surface of 4 or 5 tubes of egg media or Petrik's medium (M-58, M-58a, M-58b). Smear the surface with a platinum wire.

Replace the cork. Bore a hole in the cork, and cover with the metal cap.

Incubate two weeks or longer to obtain a growth.

(e) **Isolation and Cultivation of Tubercle Bacilli From Urine** (*Peppler and Hill*): Collect urine in 1 liter bottles containing 1.5 c.c. of a 1% solution of acriflavin and 40 c.c. of phosphate buffer pH 4.0.

Add to each bottle 5 c.c. of a 20% solution of tannic acid.

Let stand overnight in the refrigerator.

Remove the sediment with a pipette and centrifuge it at 3000 r.p.m.

Decant supernatant fluid and add an equal amount of 3% sodium hydroxide to the sediment and let it stand at 37° C. for 30 minutes.

Add 2 to 3 volumes of saline and centrifuge again.

Use the sediment for culture on Loewenstein M-60 *or* Petragnani media M-61 or for guinea pig inoculation.

BACTERIOLOGIC EXAMINATION OF AUTOPSY SPECIMENS

It is imperative that the body should not be embalmed if cultures are to be made. In order to make proper bacteriologic examinations of organs and cadavers, the outside of the organs should be seared with a hot iron and then opened with a sterile knife into the deeper parts. From this, material is removed with a sterile platinum loop.

Bacteriologic Examination of Tissue for Tubercle Bacilli*
(Mycobacterium Tuberculosis)

Grind up the granulations from curetted surfaces in either Antiformin or sodium hydroxide (normal solution) in a sterile glass mortar.

Dilute slightly.

Centrifuge at high speed for fifteen to thirty minutes.

Either make smears and stain with Ziehl-Neelsen stain, or inoculate guinea pigs. If guinea pigs are inoculated, large needles may be used, or a small skin puncture made with a sterile scalpel, and loopfuls of the sediment thrust under the skin. Seal the cut with collodion, after disinfecting with iodine.

Proceed with the examination of the guinea pig as discussed on page 1647.

ANTIBIOTIC AGENTS AND THEIR ASSAY

Antibiotic agents are defined as antimicrobial agents produced by living cells. In a Harvey Lecture,[1] Selman A. Waksman made the following observations in summarizing the chemical and biologic properties which distinguish antibiotic agents from the common antiseptics and disinfectants:

1. They are produced by living organisms, often designated as antagonists because of their ability to inhibit the growth of or to destroy bacteria and other microorganisms. Certain purely synthetic compounds have antibacterial and antifungal properties similar to those of typical antibiotic agents.

2. Their action is primarily bacteriostatic in nature, bringing about inhibition of growth; their bactericidal properties may also, however, be very pronounced.

3. They are selective in their action upon bacteria, some affecting largely gram-positive and only to a limited extent gram-negative forms, whereas others act upon certain specific bacteria within each of these groups, and only to a limited extent or not at all against other bacteria. These differences are

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*For cutting and staining tissue for tubercle bacilli, see Chapter XIV.

[1] Waksman, Selman A.: Harvey Lectures, Series XL, 1944-1945.

qualitative as well as quantitative; hence one may speak of a ''bacteriostatic'' or ''antibiotic spectrum,'' namely the selective antibacterial or antimicrobial action of an antibiotic substance.

4. The effect of the substrate upon the antibacterial activities of different antibiotic substances varies greatly. Some substrates have no effect at all upon a specific substance; others reduce the activity of the substance, due to the neutralizing effect of some constituent, such as peptone, glucose, or serum; some, like blood, may adsorb the substance, thus completely inactivating it; some substances require for their activity the presence in the medium of specific constituents, such as glucose, in order to become effective.

5. Antibiotic substances vary greatly in their chemical nature. A large number of compounds have already been isolated, although only comparatively few have so far been crystallized.

6. Antibiotic substances vary greatly in their toxicity to animals, some being highly toxic, others having virtually no toxicity at all, and most others falling between these two extremes. They also vary in their in vivo activity.

7. The mechanism of antimicrobial action of various antibiotic substances is different. Some interfere with the growth of bacteria and their cell division, others with some phase of bacterial respiration, and still others with the utilization of essential metabolites by the bacteria.

8. Many antagonistic organisms produce more than one antibiotic substance. *Pseudomonas aeruginosa* has long ago been known to produce two agents, pyocyanase and pyocyanin, distinct from one another both chemically and in their antibacterial action. *Bacillus brevis* produces gramicidin and tyrocidine; *Penicillium notatum* forms penicillin and notatin; *Aspergillus flavus* produces aspergillic acid and flavicin; *A. fumigatus* is capable of forming four substances; namely, spinulosin, fumigatin, fumigacin, and gliotoxin. The culture filtrate of an organism producing an antibiotic substance often differs, therefore, in its antimicrobial activities from that of the isolated substance.

9. Some substances are produced by different types of organisms. Penicillin and penicillin-like compounds are formed by many different strains of *P. notatum, P. chrysogenum, A. flavus, A. giganteus, A. parasiticus,* and other fungi. Gliotoxin is produced by species of Trichoderma, Gliocladium, and *A. fumigatus.* Clavacin (claviformin, patulin, and clavatin) is formed by a large number of fungi, including *A. clavatus, P. claviforme, P. patulum,* and Gymnoascus. Considerable confusion may thus result from the duplication of names for the same substance.

10. The same type of substance, when produced by different organisms or even by the same organism grown on different media or under different conditions, may show variation, both in its chemical nature and in its antibiotic spectrum, from those of the established type.

The earliest observation on antibiotics was that made by Pasteur and Joubert in 1877, followed by Cantani in 1885, by Emmerich and Low in 1899, by Vaudremer in 1913, by Much in 1924, by Fleming in 1929, and finally by Dubos in 1939. Thus, an observation on this remarkable property of certain organisms toward certain bacteria was practically overlooked for some fifty years.

Penicillin

It was Fleming who, in 1929,[2, 3] first quite accidentally observed a growth of staphylococci in a blood agar plate which had been contaminated with a mold. He saw a widely inhibited zone of bacterial growth around this mold and also noted that colonies of staphylococci within the zone became transparent as the result of varying degrees of lysis. Fleming called this substance "penicillin" and thought it might be valuable in the isolation of *Hemophilus influenzae* by preventing the overgrowth of plates by staphylococci and other penicillin-sensitive microorganisms since the penicillin had no inhibitory action on the growth of the influenza bacillus. He also suggested that it might be an efficient antiseptic to be used on areas infected with penicillin-sensitive bacteria. The classification of this mold by Fleming was not quite correct. It was Thom in this country who classified it as belonging to the *Penicillium chrysogenum notatum* series, now known as *Penicillium notatum* Westling. Florey became interested in penicillin in 1938, and in cooperation with Chain studied it carefully. They confirmed Fleming's observations, first isolated penicillin in impure form, and showed its high activity against staphylococci, streptococci, pneumococci, diphtheria bacilli, *Cl. perfringens, Cl. septicum,* and *Cl. novyi* in vitro. It was in this country that Florey found a warm welcome in 1941, and through the aid of the Department of Agriculture developed methods for increasing the production of this substance. Production on a large scale was begun by a group of American biological manufacturers.

Since the epoch-making introduction of penicillin, many other antibiotics have been added. The laboratory is often called upon to perform two tasks: (1) to determine the antibiotic concentration in the blood of the patient (antibiotic level); and (2) to determine the sensitivity of an organism, isolated from a patient, to antibiotics.

(1) Bacteriologic Control of Antibiotic Therapy

Method No. 1. Oxford Cup Method

Use *Staphylococcus aureus* PCI 209 for penicillin, *Bacillus subtilis* ATTC 6633 for streptomycin, *Sarcina lutea* PCI 1001 for Chloromycetin, Aureomycin, and Terramycin, and *Streptococcus* ATTC 8668 for Ilotycin and Magnamycin.

Enumeration of Test Organisms.—

1. Pour 21 c.c. sterile base agar M-119 into standard 100 mm. by 20 mm. Petri dishes and let it solidify. Keep for 24 hours in a refrigerator before overlaying.

2. Inoculate assay broth M-120 with the test organism and incubate for 18 to 20 hours.

3. Transfer 0.1 c.c. of this culture to 100 c.c. of melted and cooled (45° C.) seed agar M-121 or yeast beef agar H-122.

4. Pour 4 c.c. amounts of the inoculated seed agar into the Petri dishes over the base agar. Keep in a refrigerator until use.

5. Sterilize glass cylinders, 12 mm. by 8 mm., in a dry heat oven. Before use, pass them through a flame. Put 6 of these lightly, equally distanced, on the surface of each plate (No. 4). Put the plates back into the refrigerator.

6. Just before the test is carried out, prepare dilutions of the antibiotic in M/50 phosphate buffer pH 7.0 containing 0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 units or micrograms, respectively, per 0.1 c.c. of the buffer.

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[2] Fleming, A.: Brit. J. Exper. Path. **10**: 226, 1929.

[3] Fleming, A., and McLeon, I. H.: Brit. J. Exper. Path. **11**: 127, 1930.

7. Put 0.1 c.c. amounts of the dilutions prepared under No. 6 into the cups of plate No. 5. Prepare 6 such plates identically. This is the **standard series of the day.**

8. Pipette off 0.1 c.c. amounts of the plasma or serum to be tested and the same plasma or serum diluted 1:9 with M/50 phosphate buffer, and place into cups of Petri dishes prepared as under No. 5. It is advisable to use two cups on three different plates for each plasma or serum sample. On a set of three plates, three samples may be run, each of them occupying two cups of each plate. This is the **test series.**

9. Incubate standard series and test series for 18 to 20 hours.

10. Measure the zones of inhibition on the standard plates, and plot the average curve.

11. Calculate the average zone of inhibition from the test plates and find the exact value from the curve.

12. Multiply the results by 10 to obtain the number of units or micrograms per c.c. of blood or plasma.

NOTE: This is the most precise method of antibiotic level determination. The standard curve must be set up every day. When *Sarcina lutea* PCI 1001 is used for penicillin level determinations, the plates are incubated at room temperature.

Method No. 2. Serial Tube Dilution Method

Use Staphylococcus PCI 209 or *Sarcina lutea* 1001 for penicillin and Chloromycetin; *B. circulans* for streptomycin; Staphylococcus PCI 209 for Aureomycin and Terramycin; Streptococcus ATCC 8668 for Ilotycin (erythromycin) and Magnamycin level determinations.

1. Prepare culture of test organisms by growing it for 18 to 20 hours in assay broth M-120.

2. Set up four series of sterile tubes, each containing 1 c.c. of double strength assay broth M-120. Two identical series will serve as controls, two others for the test proper. If several tests are carried out with the same antibiotic, one series of controls suffices for the day's run.

3. Make up dilutions of the antibiotic containing 5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.0195, and 0.0 or no units or micrograms of antibiotic per c.c. of M/50 phosphate buffer pH 7.0.

4. Add 1 c.c. amounts of these dilutions to the tubes in the control series.

5. Make up dilutions of the plasma or serum to be tested in M/50 phosphate buffer containing 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015, and 0.0 or no c.c. of the plasma or serum per c.c. of buffer.

6. Add 1 c.c. amounts of these dilutions (5) to two series of test tubes prepared as in (2).

Note that there are 10 tubes in each control series but only 8 tubes in each test series. The last two places in the rack may be used for sterility controls: 1 c.c. of plasma or serum alone; 1 c.c. of buffer alone; 1 c.c. of broth alone; 1 c.c. of antibiotic alone.

7. Add to each tube 0.5 c.c. of the culture described under No. 1, diluted 1:100 with assay broth M-120.

8. Incubate for 18 to 20 hours.

9. Observe the tubes with the aid of a slit lamp. Find the tube containing the smallest amount of antibiotic which inhibits the growth of the test organism, in the control series.

10. Find the tube containing the smallest amount of plasma or serum which completely inhibits the growth of the test organism.

11. The two tubes described in (9) and (10) contain the same amount of antibiotic.

Calculation.—All tubes contain 2.5 c.c. Thus the amount of antibiotic added to the tube must be divided by 2.5 to obtain its concentration per c.c. The amount of serum actually contained in each c.c. of the fluid also can be obtained by dividing the amount added by 2.5. Thus the concentration of antibiotic per c.c. plasma or serum can be calculated by dividing the amount of antibiotic added to the last tube which still inhibits the growth of the organism by the amount of plasma or serum present in the last tube which shows inhibition of the test organism:

$$\text{Concentration of Antibiotic per c.c. Serum or Plasma} = \frac{\text{Amount of Antibiotic in Last Tube}}{\text{Amount of Serum or Plasma in Last Tube}}$$

Example.—

The last tube, in the control series, which completely inhibited the growth of Staphylococcus PCI 209 contained 1.25 units of penicillin. The last tube containing serum of the patient which completely inhibited the growth of this organism contained 0.25 c.c. of serum.

The penicillin level in this example is $\frac{1.25}{0.25} = 5$ units of penicillin per c.c. serum.

NOTE: Difficulties arise when two antibiotics are given to the patient. Penicillin can be inactivated by using penicillinase, about 10 units per c.c. of serum or plasma. Streptomycin will be counteracted by 0.05 gm. cysteine hydrochloride per 100 c.c. of serum or plasma. Sulfa drugs will be inhibited by 0.05 mg. p-aminobenzoic acid per 100 c.c. of serum or plasma. Such determinations, however, seldom give reliable results.

(2) Determination of Microbial Sensitivity to Antibiotics

Method 1. Tube Dilution Test

1. Grow the organism to be tested in assay broth M-119 for 18 to 20 hours.
2. Prepare a series of sterile tubes, each containing 1 c.c. of double-strength assay broth M-119.
3. Prepare dilutions of the antibiotic to be tested in M/50 phosphate buffer pH 7.0: 50, 25, 12.5, 6.25, 3.125, 1.526, 0.781, 0.39, 0.185, 0.092, 0.046, and 0.0 or no units or micrograms per c.c. of buffer.
4. Add 1 c.c. amounts of these dilutions to the tubes prepared in (2).
5. Dilute growth in (1) with assay broth M-119 1:100, and add 0.5 c.c. of this dilution to each test tube prepared in (4).
6. Prepare a control tube containing 1 c.c. of one of the antibiotic dilutions and 1 c.c. of broth.
7. Incubate for 18 to 20 hours.
8. Observe which tube contains the smallest amount of the antibiotic that still inhibits the growth of the organism.
9. Divide the total amount of antibiotic added to that tube by 2.5 to obtain the number of units or micrograms per c.c. necessary to inhibit the growth of the organism.

NOTE: Blood levels achieved by treatment with different antibiotics vary considerably. The results obtained by this method and similar methods are indicative only of the susceptibility of the organism; there is no possibility of predicting, without actual experimentation, if the necessary blood level may be obtained in a certain patient by the administration of a certain antibiotic.

Method 2. Disk Method, Difco*

Use Bacto-sensitivity disks. These disks are sterile. They must be removed from their vials with aseptic technic. The antibiotics are differentiated by the color of the disk. Concentrations are identified by the markings on each disk. Keep the vials stoppered when not in use, and store in a refrigerator.

The sensitivity test may be performed on plates of medium inoculated either with the isolated organism or with pathologic material containing the organisms.

Prepare plates of Bacto-heart infusion agar M-15, with or without added blood.

Inoculate either by smearing or streaking with a heavy suspension of the test organism or with the specimen containing the causative organisms, to obtain confluent or solid growth. Avoid excessive moisture.

Immediately place the Bacto-sensitivity disks on the surface, spacing them about 3 to 4 cm. apart. Use flamed forceps to remove the disks from the vial so as not to contaminate the remaining disks.

Incubate the plates at 35° to 37° C. for 12 hours, or until growth develops.

Examine for zones of inhibition of growth.

It is recommended that the etiological agent always be isolated, since mixed cultures might not always give dependable sensitivities.

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*Baltimore Biological Laboratories now have available BBL Sensi-Discs for determining microbial sensitivity to antibiotics.

With heavy inoculation of plates, definite zones of inhibition often occur within 5 to 6 hours after inoculation. Generally 5 or 6 sensitivity disks can be placed on a 95 mm. Petri plate. Larger plates may be used, to permit testing of the sensitivity of an organism to a larger number of antibiotics or therapeutics. Avoid crowding of disks.

A base and "seed layer" technic may be employed.

Pour 15 to 20 c.c. of sterile Bacto-heart infusion agar M-15 in sterile 95 mm. plates and allow to solidify. Use 60 c.c. for 140 mm. plates.

Incorporate the inoculum in sterile melted Bacto-heart infusion agar M-15 with or without blood, at 45° C.

Pour this inoculated seed agar on the base layer, and distribute evenly over the surface by tilting the plate to give a thin "seed layer" inoculation.

Place the disks on the surface of this layer after it has solidified.

A moderate zone of inhibition around the lowest concentration of one or more antibiotics classifies the organism as being sensitive to any or all of these antibiotics, even though there might be a difference in zone size. Resistant organisms are not inhibited in the maximum concentration of the antibiotic, while very sensitive strains show marked zones of inhibition even in the lowest concentrations. With sensitive strains, zones of inhibition are often obtained around the disk containing the lowest concentration, which are nearly as large as those obtained around the higher concentrations, due to the diffusion characteristics of the test.

Presence of zones of inhibition of growth around the disks indicates that the organism is sensitive to the antibiotic. The diameter of the zone depends not only upon the sensitivity of the organisms, but also upon the diffusability of the antibiotic and other factors which might limit the availability of the antibiotic to the organisms in the medium.

If blood agar is used for the test, one must consider hemolytic properties of microorganisms. Growth of such organisms is characterized by a lysed, cleared, or discolored background, with the presence of colonies, while zones of inhibition leave the medium unchanged, that is, with no hemolysis. Upon longer incubation, the hemolysin produced by the organism may diffuse toward the disk, giving a cleared zone, without growth, around the periphery of the zone of inhibition. The true zone of inhibition on blood agar includes both the unchanged nonhemolytic as well as the hemolytic areas showing no bacterial growth.

Frequently, isolated colonies will be observed growing within the zone of inhibition. These represent variants of the strain or culture under test. Appearance of several such resistant colonies in the zone of inhibition does not indicate the true resistance of the culture which is demonstrated by the zone of inhibition of growth.

Method 3. Rapid Method for Determining Microbial Sensitivity to Antibiotics*

Principle.—

In this method, the hemoglobin acts as a color indicator system. The characteristic bright red color of blood depends on the presence of oxyhemoglobin. When hemoglobin is reduced, the color changes from bright red to purplish red. On a Petri dish containing blood agar in a base layer and overlaid with an inoculated nutrient agar medium, the metabolism of the microorganisms results in a color change of the blood from bright red to purplish red. Where the antibiotic activity inhibits microbial metabolism, no color change of the blood occurs.

Tehnic.—

The inocula consist of clinical specimens or bacterial cultures.

Prepare Petri dishes with a thin solidified layer (5 c.c.) of heart infusion agar M-15 containing 20% citrated whole human blood. This is the base layer-hemoglobin indicator.

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*Department of Microbiology, Air University, USAF School of Aviation Medicine, Randolph Field, Texas, March, 1955. Demonstration at A. M. A. Meeting, Atlantic City, N. J., June 1, 1955.

Have at hand antibiotic-impregnated disks, which are commercially available, and tubes of melted brain heart infusion agar M-15 containing 0.8% dextrose or other appropriate nutrient media (seed layer).

Incubate the Petri dishes containing the base layer at 37° C. to pre-warm them.

Thoroughly agitate the inoculum in a tube of brain heart infusion agar, or appropriate nutrient media, which has been kept liquid in a 45° C. water bath. This produces a heavy seeding of organisms.

Pour this inoculated liquid agar as a seeded layer over the pre-warmed base layer; the latter contains the 20% whole blood.

Press color-coded filter paper disks, each impregnated with a known antibiotic, firmly on the surface of the inoculated seed layer.

Incubate the plate at 37° C. and check at 30-minute intervals for the occurrence of zones of inhibition of hemoglobin reduction surrounding the antibiotic disks.

Results.—

Prior to the appearance of visible growth, the metabolic activities of most strains of bacteria produce relatively intense reducing conditions within the medium. These conditions are reflected by a so-called ''reduction'' of oxyhemoglobin and a characteristic color change of the blood from a bright red to a purplish red. In this rapid method, readings can be made in 2 hours. The organisms reduce the oxyhemoglobin in the base layer and the bright red color of the blood turns to a purplish red except in zones around disks where the antibiotic has inhibited microbial metabolism and growth. These zones retain the bright red color of blood. Often a hemolytic organism will lyse the blood in the base layer and this plate will clear, but the zones remain easily discernible. The zones of inhibition of hemoglobin reduction are similar in size to those measured from inhibition of visible growth produced after further incubation of the inoculated plate or on control plates inoculated by the conventional surface-streaking procedure.

The incubation period for sufficient hemoglobin reduction varies with the nature of the test organism and the size of the inoculum.

This method provides the same information as the standard agar diffusion technic, but in much less time; the tests have approximately the same value in predicting the clinical outcome.

SANITARY BACTERIOLOGY

BACTERIOLOGIC EXAMINATION OF GLASSWARE OR CHINA FOR SANITARY QUALITY

Both the public and the public health officer are vitally interested in improving the unsanitary conditions prevailing in connection with dispensing of beverages. The use of single service paper cups solves the problem in some places, but we know that glasses are frequently used and are only superficially washed or disinfected between patrons. It is very probable, as has been pointed out by many observers, that beer glasses are sometimes not washed at all. Certainly, as pointed out by Calver,[1] there is a real opportunity for better control of respiratory diseases by means of improved sanitation of glasses and other eating utensils. Fellers, Levine and Harvey[2] suggest a standard method for these bacteriologic examinations. They found 18 different methods already described to be largely difficult to use and unsatisfactory. They proposed the following standard method of sampling.

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[1] Calver, H. N.: Techniques for the Bacteriological Examination of Glasses. Mimeograph. The Public Health Committee of the Cup and Container Institute, 30 Rockefeller Plaza, New York, 1935.

[2] Am. J. Pub. Health 26: No. 12, 1936.

Use medium-sized cotton swabs. Immerse in 3 c.c. of 0.85% saline in 25 c.c. culture tubes, leaving the applicator sticks protruding slightly above the plugs. Sterilize in the autoclave at 10 pounds pressure for 20 minutes, and store at low temperatures.

To secure sample, remove plug, press out excess saline from swab with a rotary movement, thoroughly swab inside and outside lips and rims of glasses. Replace applicator in culture tube and insert cotton plug. Obtain similar specimens from a number of glasses in each establishment. Label tubes. Plate on culture media within a few hours, preferably not more than two hours, as follows.

Plating.—Agitate tubes by striking against hand about 25 times. Remove plugs, squeeze out excess saline against sides of tubes, transfer entire liquid contents to Petri dish. Inoculate Loeffler's blood serum M-10 with the swab. Pour 7 c.c. agar M-113 into the Petri dish and mix thoroughly. Incubate at 37° C. for 48 hours. Record counts for individual glasses, not as averages. This method, slightly modified, may be used in the examination of dishes and silverware.

Although occasional plates may show vast numbers of colonies too numerous to count, it is better to use the entire sample for plating. This will at least give a good idea as to the sanitary quality of the glassware examined. This method is applicable to the study of bacteriologic effectiveness of mechanical dish washers and cleaning and sterilizing compounds.

"Seeing Is Believing" Method for Sanitary Examination of Restaurants

Method of Jamieson and Chen*

This ingenious method of controlling the sanitary state of eating and drinking utensils permits one at the same time to educate the personnel of restaurants in cleanliness. Swabs are taken and media are inoculated and incubated in such a way that the restaurant workers may see the results, and thus become convinced of the effectiveness of proper cleaning.

Medium M-113 is used. Keep it in flat one-ounce bottles equipped with screw-caps. Slant 4.5 c.c. of the medium in each bottle. Sand one end of the bottle to permit labeling.

Sterilize cotton-tipped applicators and keep in proper envelopes. Have on hand sterile water to moisten the swabs.

Jamieson devised a traveling laboratory consisting of these utensils and of display racks into which the bottles fit.

In restaurants 3 cups, 3 forks, 3 spoons, 3 tumblers, and 3 cream pitchers are examined. In bars and taverns, 10 glasses ready for filling and one used glass are examined.

For each eating utensil use one swab. Moisten it with sterile water. Squeeze off the superfluous water by pressing the swab against the inner walls of the bottle containing the sterile water.

Swab the inner surface and the rim of the utensils, using the side of the cotton tip and rotating it.

Inoculate with each swab one flat bottle containing the medium by rotating the swab over the surface.

Incubate the flasks in an upside-down position on the display rack at room temperature, in the establishment where the samples were taken, for 3 days.

Evaluation of results:

0 to 10 colonies:	good
11 to 50 colonies:	fair
over 50 colonies:	poor

Microbiologic Examination of Frozen Foods

The Committee on Microbiological Examination of Foods of the American Public Health Association reported[1, 2] their recommendations for methods which have proved workable as a basis for the perfecting of standard methods for frozen foods.

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*J. Milk Technol. 8: 134, 1945 and Canad. Pub. Health J. 37: 90, 1946.

[1] Am. J. Pub. Health 36: 332-335, 1946.

[2] Report issued under the chairmanship of Harry E. Goresline, Ph.D., U. S. Department of Agriculture, Washington, D. C.

In testing frozen vegetables for microbiologic content, consideration must be given to the fact that freezing storage at temperatures of approximately –10° F. (–23° C.) brings about a reduction in viable microorganisms. This may amount to roughly 50 per cent in 6 months or 75 per cent in 1 year. Thus, while a high bacterial plate count on frozen vegetables may be taken as proof of poor sanitary history, a low or moderate count does not by itself prove the opposite.

It is recommended that in the preparation of samples of frozen fruits and vegetables for microbiologic analysis a mechanical ''blender'' be employed. This has been arrived at after considerable experience with various methods of preparation on a wide variety of frozen products. In comparison with washing or hand grinding the mechanical disintegration will, as a rule, yield higher bacterial counts. However, more uniform results will be obtained, and the method is less laborious. Similarly the choice of tryptone glucose extract agar rests on the demonstrated superiority of this medium over nutrient agar or glucose agar, although glucose agar as a medium for frozen peas appears to be only slightly inferior.

Examination of Frozen Vegetables—Selection, Transportation, and Storage of Samples

Select from the lot to be examined a suitable number of packages, say 3 or 4, from each code lot of the pack. Transport in dry ice to the laboratory for analysis and place the samples in a refrigerated (0° F.) storage chest until they are to be analyzed. The temperature preferably should not rise above 0° F. (-18° C.) in the handling of the material.

Preparation of Sample.—

A. **Peas, Lima Beans, Cut Corn—Whole or Regular Cut Green Beans, etc.:**

1. **Macroscopic Examination.**—Open the package and note condition of the product, especially if ice crystals are present on the inner walls of the package and the vegetables appear to be somewhat shriveled. This condition is indicative of thawing and subsequent freezing. Record observations and any abnormalities, such as unnatural color or odor, or pink colonies of Torulae which are indicative of improper handling practices.

2. **Sampling for Plate Counts.**—The sample, if not loose-frozen, should be broken up into small units. This can be done by tapping the unopened package sharply against the table edge or by sharply striking the package with a dull instrument, being careful not to break open the package. After opening the package remove sample with sterile spoon, taking the sample from various parts of the broken out package, *e.g.*, from the center and corners.

Weigh aseptically a 50 gm. sample into a sterile borosilicate glass mechanical blender jar. Add 450 c.c. of sterile water to the jar and blend the contents for 2 minutes. If a Variac transformer is available it is advisable to increase the speed of the motor gradually and then run the blender for the required time at the 110 V. setting of the transformer.

Allow the sample to stand for 2 to 3 minutes to permit foam to subside.

Pipette 1 c.c. of the mixture into a 99 c.c. sterile water blank. Replace the cap on the dilution bottle and shake the bottle briskly 25 times through a 1 foot arc.

Pipette 1 c.c. aliquots of this mixture into each of two Petri dishes (1:1,000 dilutions) and also 0.1 c.c. aliquots into each of two more Petri dishes (1:10,000 dilution). Make a 1:100 dilution by pipetting 0.1 c.c. aliquots of the original mixture into each of two Petri dishes.

Pour melted tryptone glucose extract agar M-110, pH 7.0, cooled to 45° C., into the Petri dishes immediately, and thoroughly mix the dilution water with the agar by gently rotating the plates in a figure 8 motion with slight tilting of the Petri dish. Cool to harden, invert, and incubate at 32° C. for 4 days.

Dilutions of 1:100, 1:1,000, and 1:10,000 will usually suffice for commercially packed frozen vegetables, although further dilutions should be made if the history or the appearance of the samples warrant it.

It is of prime importance that the agar be poured immediately after the inoculum is introduced; otherwise many bacteria will adhere to the glass and an inaccurate count will result.

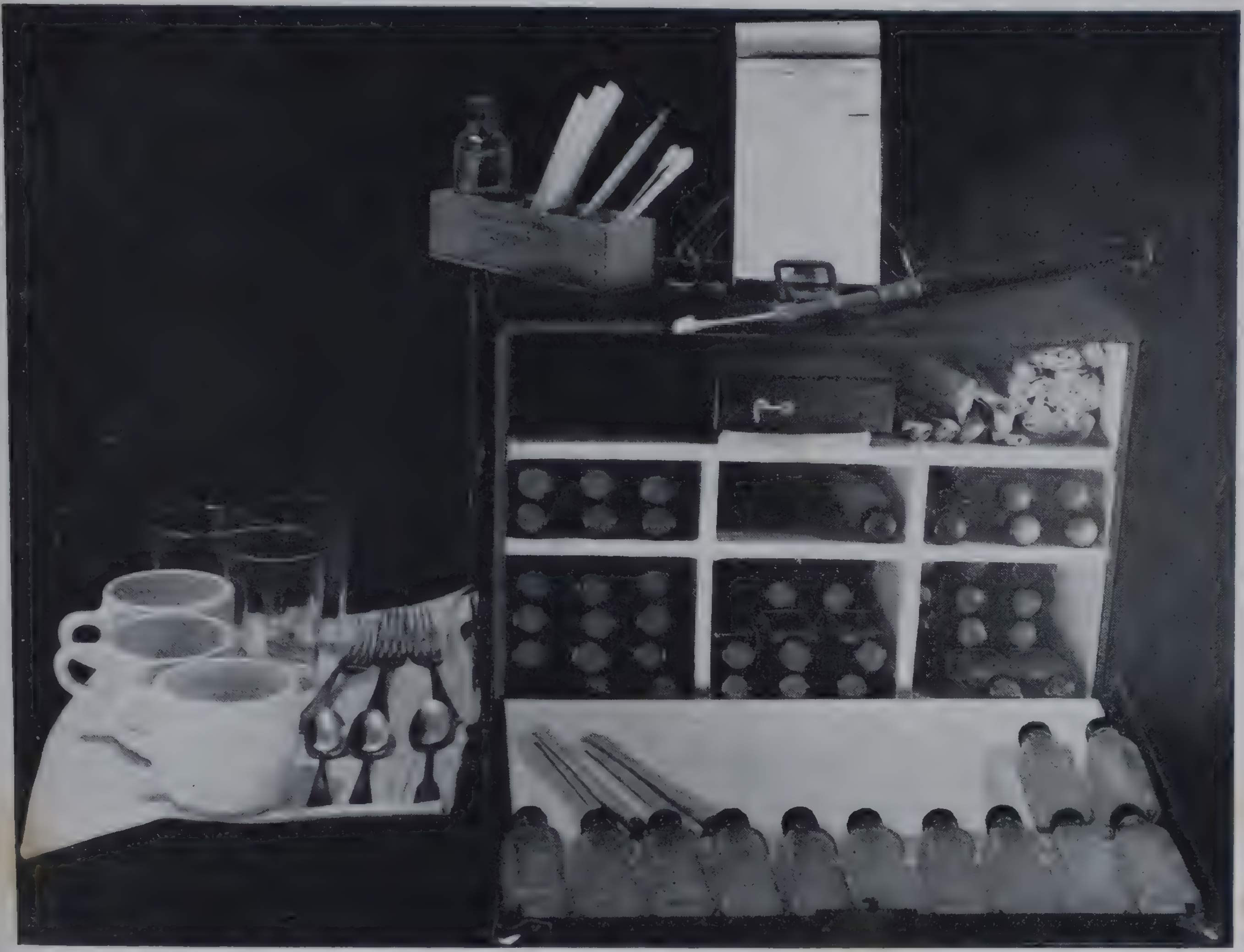

Fig. 398.—The Jamieson traveling laboratory for the "Seeing Is Believing" method of swab-slant cultures for the sanitary examination of restaurants. (Courtesy M. Jamieson.)

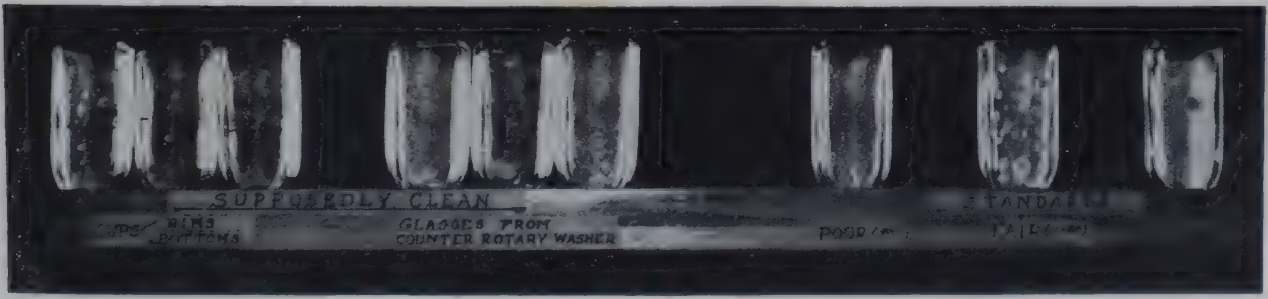

Fig. 399.—Results of examination of glasses. *Left end*, two swabbings showing prevailing conditions in one of the best restaurants. Cups washed in three-compartment machine and not dried. *Below line*, smear from swabbing bottom interior of cups—good. *Above line*, results from swabbing lips of cups—poor. *Middle three cultures* as *regularly* found from glass treated in counter rotary machine, the front of which states, "This sterilizes your glasses." Jamieson has found that this is a false statement. *Right end cultures*, arbitrary sanitary grade standard on glasses. (Courtesy M. Jamieson.)

After the incubation period is complete count the plates, using a suitable colony counter. Record results as "Plate count of microorganisms per gram."

3. **Direct Microscopic Count Method.**—The direct microscopic method has an advantage over the plate count method in that it is quicker and requires less equipment and glassware. Also it detects dead microorganisms, and indicates sanitary history, irrespective of the viable count.

Weigh 50 gm. of the vegetable (peas, lima beans, and cut corn) into a 250 c.c. iodine flask.

Add 100 c.c. water, stopper flask, and shake briskly 50 times through a 1 foot arc. Using a Breed pipette, transfer 0.01 c.c. of the washings to a microscope slide and, using a needle, spread the drop evenly over a 1 sq. cm. area of the slide. Dry and fix with heat or with methyl alcohol. Stain with methylene blue stain S-17, 18, rinse, dry, and examine under the microscope, using oil immersion. Use an ocular micrometer, such as a Whipple disk or Howard disk, with the microscope tube so adjusted that the size of the graduations is equal to 0.1 mm. (area of field 0.01 sq. mm.). Count the cells in 100 fields, and multiply the number by 20,000 to bring to the gram sample basis. Express results as "Direct microscopic estimate, microorganisms per gram."

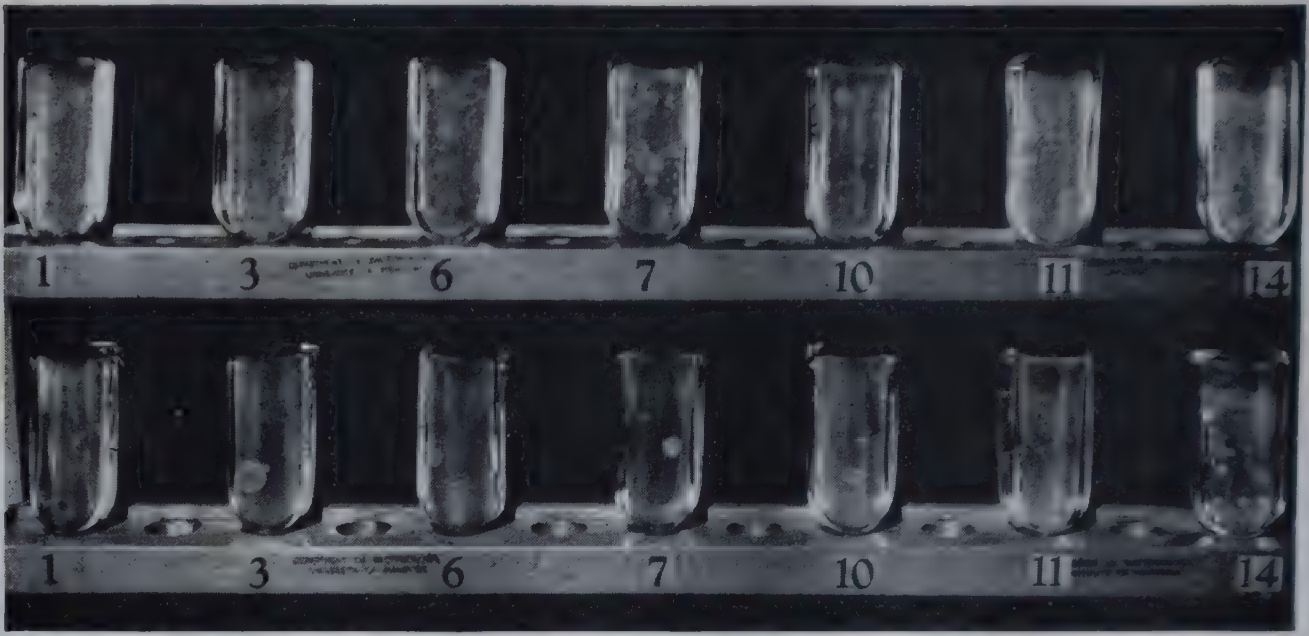

Fig. 400.—Before (top) and after (bottom) the "Seeing Is Believing" test inspired improvements in the cleaning of various types of dairy equipment. (Courtesy M. Jamieson.)

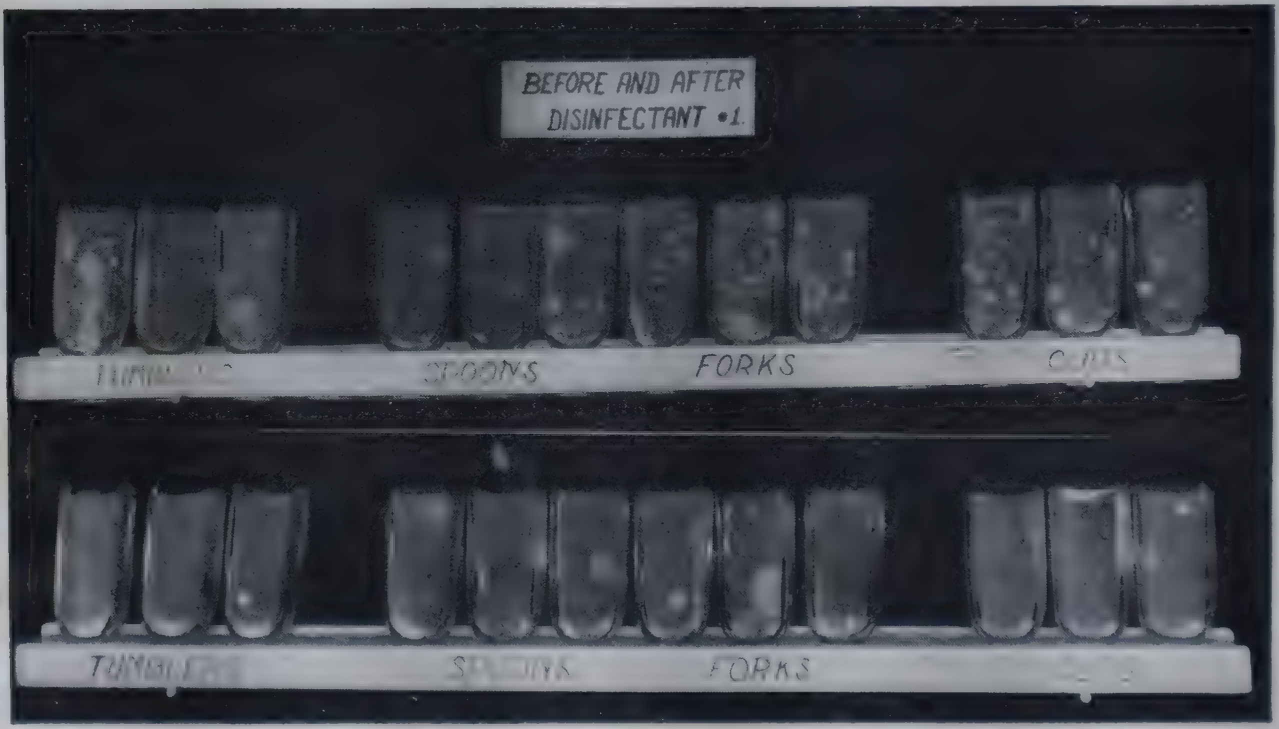

Fig. 401.—Before and after "Roccal" disinfectant and sterilizing treatment. (Courtesy M. Jamieson.)

In the direct method the following assumptions are made: (A) that all the cells are removed from the surface of the vegetable by the washing, (B) that the suspension of bacterial cells is uniform, and (C) that the drop of liquid is evenly spread over 1 square centimeter.

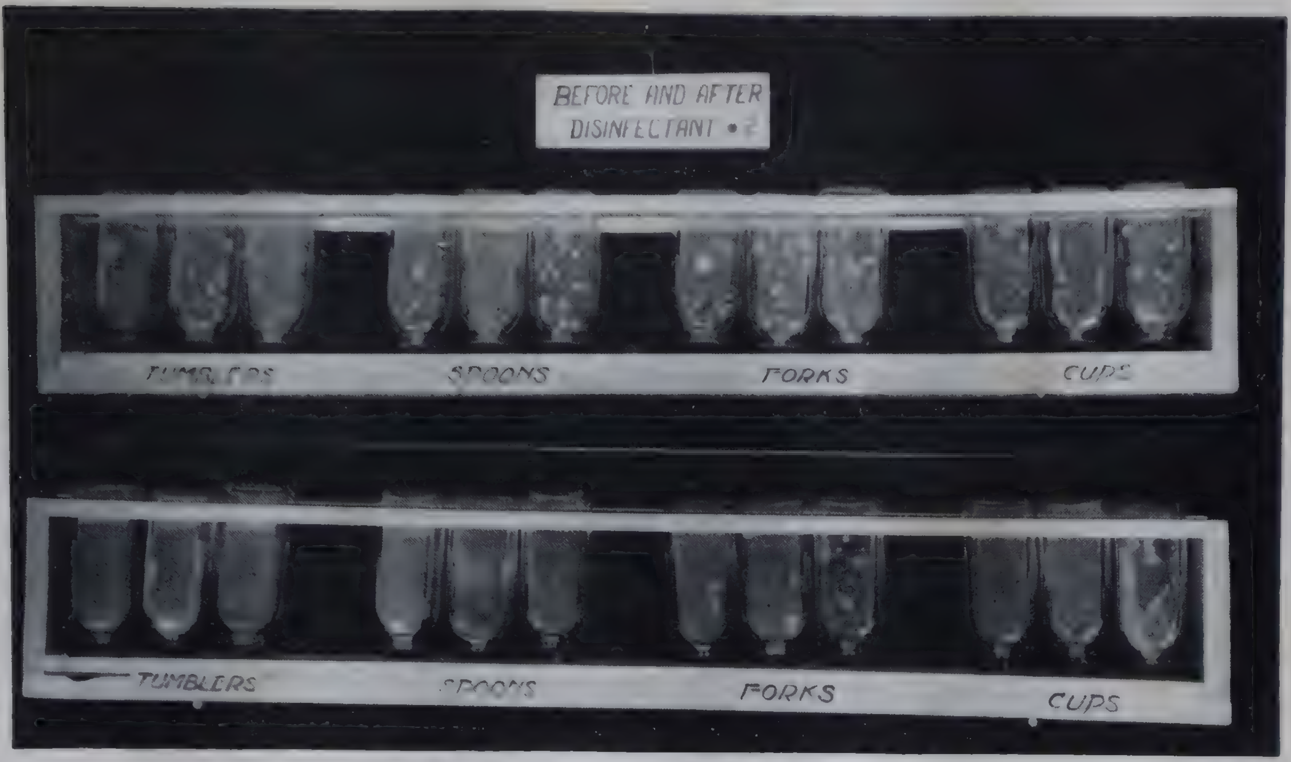
BEFORE AND AFTER DISINFECTANT #2
TUMBLERS
SPOONS
FORKS
CUPS
TUMBLERS
SPOONS
FORKS
CUPS

Fig. 402.—Before and after disinfectant "R-26" and sterilizing treatment. (Courtesy M. Jamieson.)

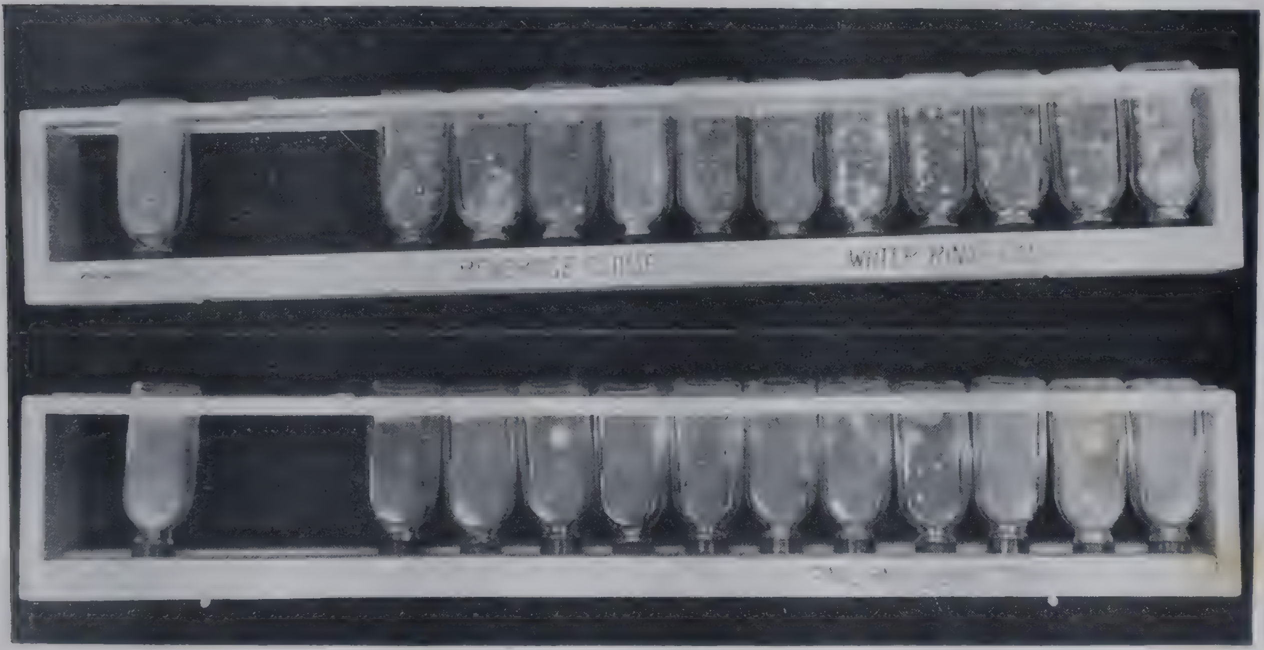

Fig. 403.—*Control,* unwashed glass direct from table in restaurant. *Others,* supposedly sanitized ready to receive beverages. Disinfectant is "Roccal." (Courtesy M. Jamieson.)

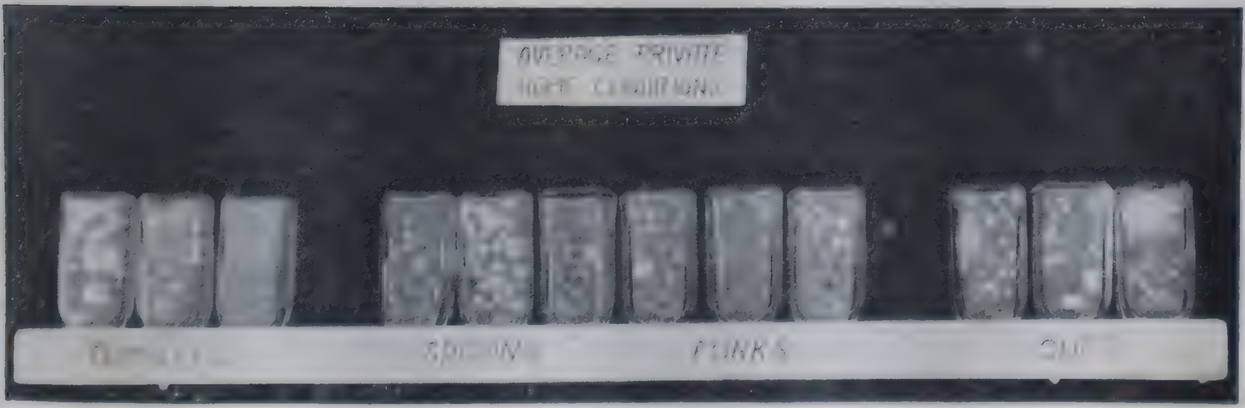
AVERAGE PRIVATE HOME CONDITIONS
SPOONS
FORKS

Fig. 404.—Average private home conditions. (Courtesy M. Jamieson.)

Interpretation of Results.—Plate counts of more than 400,000 per gram of peas or direct microscopic counts of over 1,000,000 per gram may be considered indicative of poor sanitary conditions in the plant, or poor handling practice in transit or in warehousing. In regard to other frozen vegetables it would appear that counts tend to run higher. In vegetable freezing plants plate counts of over 500,000 per gram of newly frozen produce are not encountered unless there is some degree of carelessness in the plant, such as faulty clean-up practice or prolonged holding of the material after blanching.

B. **Frozen Spinach:**

For proper comminution of spinach in a "blender" it is necessary to allow the package of spinach partially to defrost by standing at room temperature for 1½ to 2 hours. Open the package and weigh 50 gm. of the contents into a sterile borosilicate mechanical blender jar. Assemble the sample from various portions of the package, taking care to select petiole and blade portions in about the same ratio as in the sample as a whole. Add 450 c.c. sterile water. Blend for 2 minutes, and proceed as in analysis of A, peas, lima beans, etc.

C. **Frozen Broccoli and Cauliflower:**

Allow partially to defrost at room temperature. Using a sterile scalpel, cut portions from the curd and stem of several representative pieces of the vegetable. Aseptically transfer 50 gm. of these portions into the sterile borosilicate glass mechanical blender jar, add 450 c.c. sterile water, and proceed as directed for A, frozen peas, etc.

D. **Frozen Asparagus:**

Partially defrost at room temperature. Cut spears into short lengths, using sterilized scalpel. Transfer aseptically 50 gm. of the segments into a sterile borosilicate glass mechanical blender jar, add 450 c.c. sterile water, and proceed as in analysis of A, frozen peas, etc. When weighing sample portions, a number of spears should be selected and the proportion of butts and tips maintained.

The "cuts and tips" type of asparagus pack can be weighed directly into the blender cup.

Examination of Frozen Fruits

Preparation of Sample.—

Hold the package of frozen fruit at room temperature for 1 to 2 hours before opening in order partially to defrost the contents. While the fruit is still partially frozen, cut portions from various parts of the contents of the package, using a sterilized scalpel. The proportion of fruit to syrup should approximate that of the whole package. Weigh 50 gm. of fruit and syrup into a sterile borosilicate glass mechanical blender jar. Add 450 c.c. of sterile water, and blend for 2 minutes. Make a 1:1000 dilution by adding 1 c.c. of the blended mixture to 99 c.c. sterile water and make further dilutions in the usual manner. Plate 1 c.c. portions from the various dilutions on tryptone glucose extract agar M-110. Incubate for 3 days at 32° C. Count colonies under a suitable colony counter. Record results as "Plate count of microorganisms per gram."

Direct microscopic counts for molds and yeasts are made according to the methods given in *Official Methods,* A. O. A. C.[3] for microscopic analysis of tomato products. Care must be taken to distinguish between mold hyphae and fruit setae.

BACTERIOLOGIC EXAMINATION OF WATER

A. Bacterial Count

1. **Collecting the Specimen.**—The container should be a colorless glass bottle well stoppered with a cork, or with a ground-glass stopper.

Use ordinary four-ounce wide-mouth bottles with tinfoil-covered corks, and hood of heavier lead foil for collecting samples. Sterilize the bottles as usual in the dry heat oven; 10 gm. of crystallized sodium thiosulphate are dissolved in a liter of sterile water and 0.1 c.c. of the solution is added to each bottle. The bottles are then autoclaved for five minutes at 15 pounds stream pressure. Bottles should be freshly prepared for periods not exceeding two or three days, but the stock solution of sodium thiosulphate may be used over a longer period of time.

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[3] Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, ed. 5, 1940.

The bottle is sterilized in the dry heat oven at 170° C. for two hours.

The specimen should be either packed in ice or frozen. If it is frozen, it is placed in a pan of water at room temperature until it has thawed. It is kept in the ice box after the test has been made until the report has been completed.

2. **Preparation of Materials.**—Stabs of nutient agar M-13. Five or more sterile Petri dishes, preferably 5 inches in diameter.

1 c.c. pipettes, graduated in 0.1 c.c., plugged with nonabsorbent cotton, each wrapped separately, and sterilized in the dry heat sterilizer at 170° C. for two hours.

Test tubes of tap water prepared in the following manner:

Place 9 c.c. tap water in each tube. Use 5 × ⅝ inch test tubes. At the bottom of the water meniscus, make a mark with a diamond point pencil. Figure the amount of evaporation in the autoclave at 15 pounds pressure for thirty minutes in a volume of 9 c.c. and add this quantity to each tube. The amount to be added varies from 0.25 c.c. to 0.5 c.c. Plug with nonabsorbent cotton and tie paper over the top. Sterilize in the autoclave at 15 pounds pressure for thirty minutes. After the water has been sterilized, it is cooled to room temperature. If evaporation has left the fluid in each tube at the 9 c.c. mark, the water is ready for use. If the water is above or below the mark, either remove a portion or add more sterile tap water with sterile precautions until each tube contains exactly 9 c.c. sterile tap water. *Do not use distilled water.*

Sterile corks. If 5 × ⅝ inch test tubes are used, a number 5 cork will exactly fit each tube. Wrap each cork separately and sterilize in the dry heat sterilizer at 170° C. for two hours. Prepare one cork for each test tube.

3. **Technic.**—All dilutions of the water specimen must be accurate, and should be made so that no air contamination is allowed to reach the specimen.

Shake the specimen bottle vigorously 25 times before making the dilutions.

Make a 1:10 dilution of the water by adding 1 c.c. of the water specimen to 9 c.c. of sterile tap water.

After adding the 1 c.c. of water specimen, place a sterile cork carefully in the test tube to replace the cotton plug. All sterile precautions are observed during the transfer. Mix the contents of the test tube by turning upside down 25 times.

Make a 1:100 dilution of the water by adding 1 c.c. of the water specimen diluted 1:10 to 9 c.c. of sterile tap water.

Replace the cotton plug with a sterile cork, and mix as above.

Make a 1:1000 dilution of the water by adding 1 c.c. of the water specimen diluted 1:100 to 9 c.c. of sterile tap water.

Replace the cotton plug with a sterile cork, and mix as above.

Make a 1:10,000 dilution of the water by adding 1 c.c. of the water specimen diluted 1:1000 to 9 c.c. of sterile tap water.

Replace the cotton plug with a sterile cork, and mix as above.

A fresh pipette is used for each successive dilution.

Adhesive tape is placed around the side of the plate and the dilution of the water specimen marked on it. When inoculating the plates, the lid of the dish is tilted enough to admit the tip of the pipette, the pipette placed in the center of the dish, and the contents are allowed to run into the Petri dish. Do not blow through the pipette, and do not completely remove the cover of the dish. The entire process of removing the water specimen from each dilution tube, and placing in its respective Petri dish must be done very rapidly to prevent contamination with air bacteria.

Liquefy the agar M-13 and keep in a water-bath regulated at 45° C. In one tube of agar place a thermometer. This tube of agar will have to be discarded at the completion of the test. The temperature of the culture medium must not be greater than 45° C., nor less than 43° C. Higher than 45° C. will kill some of the bacteria, and lower than 43° C. will permit the medium to become solid.

In Petri dish No. I place 1 c.c. undiluted water specimen.

In Petri dish No. II place 1 c.c. of water specimen diluted 1:10.

In Petri dish No. III place 1 c.c. of water specimen diluted 1:100.

In Petri dish No. IV place 1 c.c. of water specimen diluted 1:1000.

In Petri dish No. V place 1 c.c. of water specimen diluted 1:10,000.

Pour the melted agar into each Petri dish, and mix thoroughly by rotating.

Allow to solidify.

Place upside down in the incubator for twenty-four to forty-eight hours. It is our custom to report counts at the end of twenty-four and forty-eight hours.

4. **Count as follows.**—Select the plates in which the colonies of bacteria are most evenly distributed. At least two plates should be counted. Divide the plates into sections by marking the bottoms of the dishes with a wax pencil. Count every colony on the plate.

Multiply the number of colonies counted by the dilution of the water.

Take the average, and report the result as the number of bacteria in each c.c. of undiluted water.

5. **Example.**—There were 343 colonies on the plate containing 1 c.c. of water specimen diluted 1:10.

On the 1:100 dilution of the water specimen, there were 35 colonies.

$$\begin{array}{r} 343 \times 10 = 3430 \\ 35 \times 100 = 3500 \\ 2\,\overline{)\,6930} \\ 3465 \end{array}$$

Each c.c. of undiluted water contains 3465 bacteria.

B. Examination for Evidence of Pollution

Colon bacilli are present in sewage. Examination of water for drinking purposes, therefore, is a test for colon bacilli. If colon bacilli are found there is danger of contamination of the water supply with typhoid bacilli and other intestinal organisms.

1. **Collecting the Specimen.**—The specimen is collected as described above.

2. **Preparation of Materials.**—Have on hand fermentation tubes of lactose broth M-5 made from beef extract, with inverted fermentation vials, 20 c.c. of broth per tube. Lauryl sulphate broth M-109 is preferred. Fifteen such tubes are needed.

Plate of eosin-methylene blue agar M-71, 72.

2-1 c.c. graduated pipettes.

1-10 c.c. volumetric pipette.

3. **Technic of Presumptive Test for Esch. coli.**—Using all sterile precautions, place 0.1 c.c. undiluted water specimen in 5 of the tubes of lactose broth.

Place 1 c.c. of the water in 5 of the tubes.

Place 10 c.c. of the water in 5 of the tubes.

Incubate at 37° C. for 48 hours. Examine each tube at 24 hours, and again at 48 hours. Record gas formation. Note (1) absence of gas formation; (2) formation of gas occupying less than 10 per cent of the inverted tube; and (3) the formation of gas occupying more than 10 per cent of the gas arm of the tube. If, after 24 hours' incubation, the gas formation occupies more than 10 per cent of the gas arm of the tube, this is a positive *presumptive* test for *Esch coli.* If no gas forms within 24 hours, or if the gas formation is less than 10 per cent of the arm of the fermentation tube, continue the incubation for 48 hours. The presence of any gas at all constitutes a *doubtful* presumptive test. This needs confirmation. If no gas forms within 48 hours' incubation, this constitutes a negative test.

4. **Confirmatory Tests for Esch. Coli.**—

To confirm the presence of *Esch. coli,* make transplants from the tubes showing gas formation to plates of eosin-methylene blue agar. Use the tube containing the smallest amount of water which showed gas formation. Incubate the plates at 37° C. for 18 to 24 hours. If typical colonies have developed on the plates within this period, the confirmed test is positive for *Esch. coli.* If no typical colonies develop within 24 hours, reincubate for 24 hours.

Colon bacilli grow black, opaque, and iridescent with metallic sheen on eosin-methylene blue agar.

Confirm results by transferring colonies to differentiating media as listed under "Coliform Organisms," pages 1536 ff.

Report results as: *Esch. coli* present (absent) in five samples of ______ c.c. water.

5. **Culture of Typhoid Bacilli and Other Salmonellae From Water.—**

Inoculate 5 tubes of double-strength selenite-M (M-78) with the water, adding equal amounts to the fluid.

Incubate for 24 hours and make streak inoculations from each tube on one SS plate M-67.

Pour one bismuth sulphite plate M-70 with 1 c.c. of the contents of each tube.

6. **Detection of Cholera Vibrios in Water.—**

Inoculate samples of water into alkaline sea-salt fluid M-98, using inocula of about $\frac{1}{10}$ of the volume of the fluid in the tubes or flasks.

Incubate for 24 hours.

Plate from each tube to Aronson's M-74 and Wilson and Reilly M-75 medium.

7. **Detection of Clostridium Perfringens.—**

This organism, present in nearly every bowel, is a suitable indicator of fecal contamination of the water. Not more than one organism is admissible per 200 c.c. of water.

Add to bismuth sulphite agar M-70, while hot, enough agar to make the agar content 3 per cent.

Cool to 60° C.

Mix equal amounts of water and bismuth sulphite medium.

Pour into large plates, 15 cm. in diameter, in a thick layer.

Allow to solidify and then cover with a layer of medium without water.

Incubate for 72 hours and observe for black, lens-shaped colonies.

C. Membrane Filter Procedure for Testing for Coliform Organisms in Water

This procedure for bacteriologic examination of water makes use of the Millipore filter.* The subcommittee on Standard Methods for the Examination of Water and Sewage of the American Public Health Association found that there is no readily apparent relationship betweeen results obtained by this method and the standard dilution tube procedure. Their investigation indicated that the two procedures do not measure precisely the same group of microorganisms; the sanitary significance of the different results must be determined.

A membrane filter procedure has been developed as a tentative method, but it cannot be considered as an acceptable substitute for the dilution tube method at the present time.

The full tehnic is given on pages 395 to 404 of *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes*, ed. 10, 1955, American Public Health Association, Inc.

D. Chemical Analysis of Water

For the chemical analysis of water, a liberal sample of fresh water should be submitted to the laboratory. This sample, at least one gallon, should be representative of the entire water supply which is to be examined. Procedures described here are largely those recommended by the U. S. Army and the American Public Health Association.

Physical Examination.—

1. Turbidity
2. Color
3. Odor
4. Reaction in terms of hydrogen ion concentration

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*Obtainable from the Millipore Filter Corporation, Watertown 72, Mass., who also supply a pamphlet with full information for its use.

Chemical Examination.—

1. Total residue
2. Loss on ignition
3. Nitrogen cycle
4. Alkalinity
5. Total hardness
6. Oxygen consuming power
7. Chlorides
8. Sulphates
9. Sodium and potassium
10. Total iron
11. Silica
12. Aluminum
13. Calcium
14. Magnesium
15. Residual chlorine

Most of the equivalents in water chemistry calculations are computed on a parts per million basis. Suggestions for interpreting the quality of the water are given in a chart at the end of this section (page 1674).

Turbidity

Turbidity of water is due to suspended matter in the water. To determine turbidity of the specimen, compare with standard suspensions or standard discs ranging from 5 to 100 parts per million. Report turbidity as less than 5 ppm, or between 10 and 15 ppm, or as 5 ppm, etc.

Standard Suspensions.—

Place 5 grams of Fuller's earth in 1 liter of distilled water.

Agitate intermittently for 1 hour and allow to stand for 24 hours.

Withdraw the supernatant liquid without disturbing the sediment.

Dilute successive portions of the sediment with distilled water to prepare the various standards ranging from 5 to 100 parts per million. Use a candle turbidimeter for standardization.

Standard Disks.—

Permanent turbidity standard disks are available from most large chemical supply houses. The disks may be used in place of the turbidity standard suspensions.

Combination water color and turbidity test sets, U. S. Geological Survey Standard, may be purchased from any large chemical supply company.

Technic.—

The standard unit of turbidity is that produced by 1 part per million of silica in distilled water.

Thoroughly shake the water to be tested. Pour a portion of it into a 125 c.c. glass stoppered bottle and shake.

Shake the standard suspensions and compare in a dark box turbidimeter. Record turbidity in parts per million. Permanent standard disks may be used in place of the standard suspensions. If the Jackson candle method[1] is used, report in turbidity units.

Color

Color disks, calibrated from a platinum-cobalt color scale, may be purchased from any large chemical supply house. Place these glass disks on the end of a metal tube containing distilled water.

Place the water to be tested in a second tube.

Compare and record the color number.

Odor

Odor of water specimens may be described as musty, aromatic, fishy, moldy, etc., and as negative, faint, moderate, strong.

If an odor is detectable in the sample, the odor concentration must be determined.

Place 200 c.c. of the sample in a 500 c.c. flask.

Add 200 c.c. of water free of odor. This is a 1 to 2 dilution.

Mix without unduly exposing to the air. Do not agitate unnecessarily.

Add 200 c.c. of this dilution to 200 c.c. of water free of odor in another 500 c.c. flask. This is a 1 to 4 dilution.

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[1] Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, ed. 10, 1955, American Public Health Association, Inc., p. 207.

Mix, and continue with the dilutions to obtain 1:8, 1:16, 1:32, and further dilutions, until the second dilution beyond which the odor of the specimen is no longer detectable. If the odor is very strong, omit some of the lower dilutions.

Shake and examine each flask in order, beginning with the strongest dilution and proceeding to the weakest until an odor is just detectable in one of the flasks. This is the threshold odor and is recorded as the odor concentration.

Report the *odor concentration* as the reciprocal of the dilution; e.g., one, two, four, eight.

Hydrogen Ion Concentration

Determine the pH colorimetrically by using a series of indicators and comparing with prepared standards of definite hydrogen ion concentration. The indicators most commonly used are brom thymol blue and phenol red. Use 0.5 c.c. of the indicator to 10 c.c. of the water. See pages 30 ff. for detailed methods of determining hydrogen ion concentration.

Total Residue—Loss on Ignition

Shake the sample thoroughly but do not filter.

Place 100 c.c. of the sample in a weighed platinum dish.

Evaporate on a water or steam bath.

Dry the residue at 103° to 105° C. for 1 hour.

Weigh the crucible and contents. Subtract the weight of the platinum dish from the total weight to obtain the total solids or residue on evaporation. This gives the residue at 103° C. Residues obtained from waters low in organic matter but relatively high in iron may be conveniently used for the determination of iron.

Loss on Ignition and Fixed Residue.—Ignite the residue in the platinum dish at low red heat. Note any significant change in color or odor. Allow the dish to cool.

Moisten the residue with distilled water, then dry at 103° C., cool, and weigh. Subtract this figure from the weight recorded as "residue at 103° C." This figure represents the *loss on ignition*. The weight of the residue is recorded as the *fixed residue*.

For the determination of total solids in waters of high salt concentration, see *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes,* ed. 10, 1955, American Public Health Association, Inc.

Nitrogen Cycle

Ammonia Nitrogen: Albuminoid Nitrogen.—

Distilling Apparatus.—Use a glass flask with a vertical condenser, so arranged that the distillate drops directly into the Nessler tube (50 c.c.). The end of the condenser should be fitted with a glass cup into which the Nessler tubes are inserted when collecting the distillate. Use tin foil to completely cover the rubber stopper.

Reagents.—

Ammonia-Free Water.—

Nessler's Solution.—

Dissolve 50 gm. of potassium iodide
in 35 c.c. of cold ammonia-free water.

Add saturated solution of mercuric chloride until a slight precipitate forms and persists.

Add about 400 c.c. of 9N sodium hydroxide which has been clarified by sedimentation.

Dilute to 1000 c.c. with ammonia-free distilled water. Allow the solution to clarify and decant the clear supernatant fluid.

Stock Ammonium Chloride Solution.—

Dissolve 3.818 gm. ammonium chloride in ammonia-free water in a liter volumetric flask and dilute to 1000 c.c. with ammonia-free water. 1 c.c. contains 1 mg. N.

Standard Nitrogen Solution.—

Dilute 10 c.c. of the stock ammonium chloride solution to 1000 c.c. in a liter volumetric flask, using ammonia-free water.

1 c.c. contains 0.01 mg. of N, equivalent to 0.01288 mg. of NH_4.

Phosphate Buffer Solution, pH 7.4.—

Dissolve 14.3 gm. of monobasic potassium phosphate (KH_2PO_4)
and 90.15 gm. of dibasic potassium phosphate ($K_2HPO_4.3H_2O$) in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

Alkaline Potassium Permanganate Solution.—

Boil 1,200 c.c. of distilled water for 10 minutes to expel the ammonia. Add 16 gm. of potassium permanganate and stir until dissolved.

Add 800 c.c. of a clarified 9N sodium or potassium hydroxide solution.

Dilute to 2,500 c.c. with ammonia-free water.

Concentrate to 2 liters.

Determine the ammonia in this reagent using Nessler reagent. Use this figure as a correction factor.

Technic.—

Free the apparatus from ammonia by boiling the water until the distillate shows no signs of ammonia by the Nessler test.

Empty the flask and measure into it 500 c.c. of the water to be tested.

Add 10 c.c. of phosphate buffer.

Distill at the rate of not more than 10 c.c. nor less than 6 c.c. per minute.

Collect in 50 c.c. portions in four Nessler tubes and dilute to 50 c.c. with ammonia-free water.

Prepare standards in similar tubes. Standards must contain 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 c.c. of the standard nitrogen solution, covering the range of nitrogen expected in the water sample. Dilute the standards to 50 c.c. with ammonia-free water.

Now add 50 c.c. of alkaline potassium permanganate solution to the contents of the distillation flask, after the ammonia has been removed, and continue distillation until five 50 c.c. portions are recovered.

Nesslerize both the standards and the nine portions of distillate by adding 1 c.c. of Nessler's reagent to each tube.

Allow the tubes to stand not less than 10 minutes.

Compare with the standard tubes.

Calculation.—

The sum of the readings of the first four tubes × 2 × 0.01 = the ammonia nitrogen content in parts per million.

The sum of the readings of the last five tubes × 2 × 0.01 = the albuminoid nitrogen content in parts per million.

Nitrate and **Nitrite Nitrogen.—**

Nitrite N.—

Reagents.—

Sulphanilic Acid Solution.—

Dissolve 8 grams of sulphanilic acid in 5N acetic acid and dilute to 1 liter with 5N acetic acid in a liter volumetric flask.

a-**Naphthylamine Hydrochloride** (or **Acetate**).—

Dissolve 5 grams of *a*-naphthylamine hydrochloride or acetate in 5N acetic acid in a liter volumetric flask and dilute to 1 liter with 5N acetic acid.

Stock Sodium Nitrite Solution.—

Dissolve 1.1 gm. silver nitrate in nitr*ite*-free water. Precipitate the silver with sodium chloride solution. Dilute to 1000 c.c. with distilled water.

Nitrogen Standard.—

Dilute 100 c.c. the stock sodium nitrite solution to 1000 c.c. in a liter volumetric flask.

Mix and transfer 50 c.c. to a liter volumetric flask.

Dilute to 1 liter with sterilized nitrite-free water.

Add 1 c.c. of chloroform and store in a sterilized bottle.

1 c.c. contains 0.0005 mg. N and is equivalent to 0.001642 mg. NO_2.

Aluminum Hydroxide.—

Electrolize ammonia-free water with aluminum electrodes. Wash the precipitate until it is free from chlorides, ammonia, and nitrite.

Or, dissolve 125 gm. of potassium of ammonium alum in one liter of distilled water. Precipitate the aluminum by adding the ammonium hydroxide cautiously. Wash until free from chlorides, nitrites, and ammonia.

Technic.—

Place in a standard Nessler tube 50 c.c. of the sample, decolorized, if necessary, with aluminum hydroxide.

At the same time prepare standards in Nessler tubes covering the range of the suspected nitrite N. Use 0.0, 1, 2, 3 c.c., etc., of the standard solution.

Add 1 c.c. of sulphanilic acid solution

and 1 c.c. of *a*-naphthylamine hydrochloride solution to the sample and the standards.

Mix thoroughly.

Allow to stand not less than 10 minutes.

Compare the unknown with the standard solutions. If the color of the sample is deeper than that of the highest standard prepared, repeat the test using diluted samples.

Nitrate N.—

Reagents.—

Phenoldisulphonic Acid Solution.—

Dissolve 25 gm. of pure white phenol
in 150 c.c. of concentrated sulphuric acid.
Add 75 c.c. of fuming sulphuric acid (15% free SO_3).
Stir well.
Heat for 2 hours at 100° C.

Potassium Hydroxide Solution, Approximately 12N.—

See pages 23 ff. for normal solutions.

10 c.c. of this solution should neutralize 4 c.c. of phenoldisulphonic acid.

Standard Nitrate Solution.—

Dissolve 0.7216 gm. of pure recrystallized sodium nitrate in 1 liter of distilled water.

Evaporate 50 c.c. of this solution to dryness on the water bath. Moisten the residue quickly and thoroughly with 2 c.c. of phenoldisulphonic acid and rub with a glass rod to insure intimate contact.

Dilute to 500 c.c. with distilled water.

1 c.c. contains 0.01 mg. of N, equivalent to 0.04427 mg. NO_3.

Standard Silver Sulphate Solution.—

Dissolve 4.397 gm. of silver sulphate in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

1 c.c. is equivalent to 1 mg. of Cl.

Technic.—

If nitrite N is present in excess of 1 part per million, remove it by heating the water specimen with a few drops of hydrogen peroxide. Subtract the nitrite N equivalent from the final reading.

If the color is greater than 10 (see under *Color*), decolorize with 2 or 3 c.c. of aluminum hydroxide solution.

Filter and add enough sulphuric acid to make neutral to phenolphthalein.

If chlorides are present in concentrations above 3 mg. in the sample (see *Chloride* results), remove all but 0.1 mg. of chlorine by adding standard silver sulphate. Excess silver sulphate will cause loss of nitrite N.

Filter and evaporate to dryness.

Heat the dish for a few minutes.

Add 2 c.c. of phenoldisulphonic acid and rub with glass to insure intimate contact.

Dilute with distilled water.

Add potassium hydroxide solution slowly until the maximum yellow color is developed.

Transfer the solution to a Nessler tube, filtering if necessary, and dilute to 50 c.c. with distilled water.

Prepare standards using varying quantities of the standard nitrate solution (0.01 mg. of N, 0.02, etc.). Treat the standards in a similar manner.

Compare the unknown solutions with the standards and record the results.

Alkalinity

Reagents.—

Phenolphthalein Indicator.—

Dissolve 5 gm. of phenolphthalein in 1 liter of 50 per cent alcohol. Neutralize with N/50 sodium hydroxide. The alcohol should be diluted with distilled water which has been boiled and cooled quickly.

Methyl Orange Indicator.—

Dissolve 5 gm. of methyl orange in 1 liter of distilled water. Keep the solution in the dark.

Technic.—

With Phenolphthalein.—

Add 4 drops of phenolphthalein indicator to 50 or 100 c.c. of the water sample in a white porcelain casserole or in an Erlenmeyer flask over a white surface. If the solution becomes colored, hydroxide or normal carbonate is present.

Titrate with N/50 sulphuric acid from a buret until the color disappears. This is the end point.

Calculation.—C.c. of N/50 sulphuric acid used × 20 = the phenolphthalein alkalinity in parts per million of calcium carbonate if 50 c.c. of the water sample were used. If 100 c.c. were used, multiply by 10 instead of by 20.

With Methyl Orange.—

Use 2 drops of methyl orange indicator to 50 or 100 c.c. of the water sample. The solution develops a yellow color in the presence of hydroxide, normal carbonate, or bicarbonate. Titrate with N/50 sulphuric acid to the first faint pink color; that is, until the yellow color is no longer a pure yellow. Calculate as for phenolphthalein.

Bicarbonate (HCO_3) is present if the alkalinity to phenolphthalein is less than one-half of the alkalinity to methyl orange. The alkalinity to methyl orange is due entirely to bicarbonate if there is no phenolphthalein alkalinity. The bicarbonate in terms of $CaCO_3$ is equal to the methyl orange alkalinity minus twice the phenolphthalein alkalinity; or if no phenolphthalein alkalinity exists, it is equal to 1.22 times the bicarbonate expressed in terms of $CaCO_3$ (methyl orange alkalinity).

Carbon dioxide (CO_2) as bicarbonate is equal to 0.88 times the bicarbonate expressed in terms of $CaCO_3$.

Half-bound CO_2 is equal to 0.44 times the bicarbonate expressed in terms of $CaCO_3$.

Total Hardness

(Soap Method)

Reagents.—

Stock Standard Soap Solution.—

Dissolve 100 gm. of pure powdered castille soap in 1 liter of 80 per cent alcohol. Allow to stand overnight. Decant. This solution is approximately ten times stronger than needed for the working solution.

Working Soap Solution.—

Titrate the standard soap solution against a standard calcium chloride solution, 1 c.c. of which is equivalent to 1 mg. of calcium carbonate. Dilute the stock standard solution with 80 per cent alcohol in accordance with the titer so that 1 c.c. is equivalent to 1 c.c. of the standard calcium chloride solution.

Standard Calcium Chloride Solution.—

Dissolve 0.5 gm. of pure calcite with a small amount of dilute hydrochloride acid. Wash down with carbon-dioxide-free distilled water and neutralize with ammonium hydroxide. Add a small amount of ammonium hydroxide to produce slight alkalinity. Dilute with carbon-dioxide-free distilled water to 500 c.c. 1 c.c. is equivalent to 1 mg. of calcium carbonate.

Technic.—

Place 50 c.c. of the water to be examined in an 8 ounce glass-stoppered bottle.

Add from a buret the working standard soap solution, using small amounts (0.5 c.c.) at a time. Shake vigorously after each addition. Continue adding the soap solution until a strong permanent lather, which stands 5 minutes with the bottle laid on its side, is secured. This is the end point. When approaching the end point, reduce the addition of the soap solution to 0.1 to 0.2 c.c. at a time.

A blank titration should be made using distilled water against the soap solution. The result of this titration is referred to as the "lather factor."

Calculations.—Subtract the lather factor from the titration results and multiply by 20 to obtain the total hardness in terms of calcium carbonate in parts per million.

If a hardness test requires more than 15 c.c. of soap solution, dilute an aliquot of the water to 50 c.c. with carbon-dioxide-free distilled water so that the final lather point will be less than 15 c.c. Deduct the lather factor (blank) and multiply the result by 20 then by the dilution factor to obtain the correct hardness value.

If the water is acid, render it neutral to methyl orange using N/50 sodium carbonate solution before determining the total hardness.

Oxygen Consuming Power

Reagents.—

Dilute Sulphuric Acid.—

Add 1 part of concentrated sulphuric acid to 3 parts of distilled water, cooling under running water while adding the acid.

Ammonium Oxalate Solution.—

Dissolve 0.888 gm. of ammonium oxalate, c.p., in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

1 c.c. is equivalent to 0.1 mg. of oxygen. This solution is stable for about one month.

Standard Potassium Permanganate Solution.—

Dissolve 0.4 gm. potassium permanganate in 1 liter of distilled water.

Add 10 c.c. of this solution and 10 c.c. of dilute sulphuric acid solution to 100 c.c. of distilled water in a flask.

Digest for 30 minutes in boiling water.

Add 10 c.c. of ammonium oxalate solution.

Titrate to a pink color with the standard potassium permanganate solution to destroy the oxidizing capacity of the distilled water.

Again add 10 c.c. of the oxalate solution and titrate with the potassium permanganate solution.

Adjust the permanganate solution so that 1 c.c. is equivalent to 1 c.c. of the oxalate solution, or to 0.1 mg. of available oxygen. Preserve in a dark glass bottle.

33 Per Cent Sodium Hydroxide Solution.—

Dissolve 500 gm. of sodium hydroxide in 1 liter of distilled water. Allow to settle and decant the clear supernatant fluid.

Technic.—

If the water to be tested is relatively pure, use 100 c.c.

If sewage is being examined, dilute 1 to 10 c.c. quantities of the sample to 100 c.c. with distilled water.

The volume of the sample plus the diluent must be 100 c.c. and the quantity of the sample must be such that an excess of not less than 5 c.c. of the permanganate remains after digestion.

Add 10 c.c. of permanganate solution to the sample in a flask.

Add 10 c.c. of dilute sulphuric acid solution.

Digest for 30 minutes in a hot water bath in such manner that the entire amount of sample plus reagents is submerged.

Add 10 c.c. of the oxalate solution.

Titrate with permanganate solution to a faint pink color.

Digest a blank of distilled water and the reagents used, decolorize with the oxalate, and titrate back with the permanganate.

Calculations.—

$$\frac{\text{Titration results} - \text{blank}}{\text{c.c. sample used}} \times 100 = \text{oxygen consumed, expressed in parts per million.}$$

Chlorides

Reagents.—

Standard Sodium Chloride Solution.—

Dissolve 16.4858 gm. of pure fused sodium chloride in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

Transfer 100 c.c. of this solution to a liter volumetric flask and dilute to 1000 c.c. with distilled water.

1 c.c. contains 1 mg. of chloride radical.

Standard Silver Nitrate Solution.—

Dissolve 2.4 gm. of silver nitrate crystals in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

Titrate against the standard sodium chloride solution using potassium chromate indicator. Adjust so that 1 c.c. of the solution is exactly equivalent to 0.5 mg. of Cl radical.

Potassium Chromate Indicator.—

Dissolve 50 gm. of neutral potassium chromate in a small amount of distilled water. Add silver nitrate solution to produce a slight red precipitate. Allow to stand for 1 or 2 days and filter and dilute the filtrate to 1 liter with distilled water.

Aluminum Hydroxide.—

See page 1666.

Technic.—

If the sample has a color value greater than 30 (see *Color*), decolorize by shaking it thoroughly with aluminum hydroxide, 3 c.c. to 500 c.c. of the sample. Filter. Make the determination in aliquot portions of the clarified sample.

Before titrating the chloride, add 2 drops of phenolphthalein indicator. If a red color appears, neutralize with 0.02 N sulphuric acid. If the water is acid to methyl orange, neutralize with 0.02 N sodium carbonate. If the sample contains hydrogen sulphide, acidify with sulphuric acid, boil a few minutes, cool, neutralize with sodium bicarbonate, and restore the original volume of the sample. If the amount of chloride appears to be more than 50 parts per million, use 25 c.c. or less of the sample and dilute to 50 c.c. with distilled water.

If the chloride is less than 10 parts per million, add a previously measured amount of standard sodium chloride solution and make corrections accordingly in the final calculations.

Add 1 c.c. of potassium chromate indicator to 50 c.c. of the prepared sample in a 6-inch white porcelain dish.

Titrate with standard silver nitrate solution. The end point is very sharp when viewed by yellow electric light. The first very faint reddish color is the end point.

Calculations.—

$$\frac{1000}{2 \times \text{c.c. sample titrated}} \times \text{c.c. silver nitrate solution used} = \text{chloride in parts per million.}$$

Sulphates

Evaporate 250 c.c. of sample to dryness. Before the sample is completely dry add 1 c.c. of 1:2 hydrochloric acid. Remove the silica as previously described.

Add a hot solution of barium chloride to the hot filtrate and warm it, stirring at intervals, for one-half hour or until the precipitate settles readily and leaves a clear supernatant fluid.

The precipitate is barium sulphate.

The filtrate can be used to determine sodium and potassium.

Filter, dry, ignite the precipitate as barium sulphate, of which 41.14 per cent is equal to the content of SO_4.

Sodium and Potassium (as Sodium)
By Calculation

To determine sodium and potassium by calculation, all basic and acid radicals must have been determined. Multiply these by the following factors:

BASIC RADICALS	FACTOR	ACID RADICALS	FACTOR
Calcium	0.04991	Bicarbonates	0.01639
Magnesium	0.08224	Sulphates	0.02082
		Chlorides	0.02828

If the sum of the products of the basic radicals is greater than that of the acid radicals, sodium and potassium are not present. But if acid radicals are greater than the basic radicals, divide the difference by the factor for sodium, 0.04348. The quotient represents the amount of sodium as Na and K in parts per million.

Total Iron

Reagents.—

Standard Ferric Iron Solution.—

Dissolve 0.7022 gm. of crystallized ferrous ammonium sulphate ($FeSO_4(NH_4)_2SO_4.6H_2O$) in 50 c.c. distilled water and 20 c.c. of concentrated sulphuric acid.

Warm the solution and add potassium permanganate solution until the iron is completely oxidized. Dilute to 1 liter with distilled water.

1 c.c. contains 0.1 mg. Fe.

Dilute Hydrochloric Acid.—

To 2 volumes of distilled water add 1 volume of concentrated hydrochloric acid, specific gravity 1.2. This is an approximate 3N solution.

Potassium Permanganate Solution.—

Dissolve 6.3 gm. of potassium permanganate in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water. This is an approximate N/5 solution.

Potassium or Ammonium Thiocyanate.—

Dissolve 2 gm. potassium or ammonium thiocyanate in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water.

Hydrochloric Acid.—

Concentrated hydrochloric acid free from iron.

Nitric Acid.—

Nitric acid, specific gravity 1.42, free from iron.

Nitric Acid.—

Nitric acid, specific gravity 1.195. Use 382 c.c. of this concentrated nitric acid per liter of solution. This is approximately a 6N solution.

Technic.—

Evaporate 100 c.c. (or less) of the sample to dryness, or use the residue on evaporation. With silt-bearing samples of water the quantity of iron is sometimes so great that it is necessary to use as little as 10 c.c. of the sample. In such cases add 5 to 10 c.c. of concentrated hydrochloric acid to the sample. If the sample contains much organic matter, destroy the organic matter by ignition so that the iron dissolves only with difficulty.

Heat the dish to drive off excess of acid if the residue was not ignited.

Cool and add 0.8 to 1.0 c.c. of 3N hydrochloric acid.

Warm on the water bath taking care to avoid evaporation to dryness by adding small quantities of distilled water from time to time.

Rinse the hot solution into a 50 c.c. Nessler tube, filtering if necessary.

Add 1 to 2 drops of potassium permanganate solution. If the color of permanganate does not persist for at least 5 minutes, add more permanganate solution, drop by drop.

Cool and dilute to the mark with distilled water.

Prepare iron standards by placing in matched Nessler tubes 0.05 c.c., 0.10 c.c., etc., up to 4 c.c. (or more), of standard iron solution covering the range of standards required for the determination at hand. Dilute each to about 40 c.c. with distilled water.

Add 0.5 c.c. of 3N hydrochloric acid and 1 or 2 drops of potassium permanganate solution to each tube. Dilute to the mark.

Add 5 c.c. of thiocyanate solution to the unknown and to the standards. Mix each tube thoroughly and compare immediately.

If the sample is low in organic matter, boil 50 c.c. with 5 c.c. of 6N nitric acid for 5 minutes, add 3 drops of permanganate solution, and cool. Add 5 c.c. of thiocyanate solution and compare immediately with standards prepared from the standard iron solution with 6N nitric acid instead of with 3N hydrochloric acid.

Silica

Gravimetric Method

Evaporate 500 c.c. of the sample in a platinum crucible. When the residue is nearly dry add 1 c.c. of dilute hydrochloric acid (1:2), moisten the sides of the dish, and evaporate to dryness. If much organic matter is present, char in a radiator.

Add a few drops of hydrochloric acid, dissolve in hot water, and filter.

Ignite and weigh the insoluble residue.

Add 2 drops of concentrated sulphuric acid and a little hydrofluoric acid, volatilize the acids, ignite, and weigh again.

Report the loss in weight as silica (SiO_2). A weight of nonvolatile matter exceeding 0.5 mg. should be analyzed.

Aluminum

Use the filtrate from the insoluble residue. Heat to boiling, oxidize with concentrated nitric acid or bromine, and concentrate to 100 c.c.

Add ammonium hydroxide in slight excess, boil one minute, and filter.

Ignite the precipitate and weigh as ferric and aluminum oxides.

Calculate the iron determined in terms of ferric oxide and subtract this from the weight of the ignited oxides. Aluminum is 53 per cent of the remainder.

If much phosphate is present, it must be determined in a separate sample, and the amount of P_2O_5 subtracted from the ignited oxides.

Calcium

Concentrate the filtrate obtained from the determination of aluminum to 100 c.c.

Add an excess of fresh saturated solution of ammonium oxalate, little by little, to the hot ammoniacal solution. Keep the solution warm.

Stir at intervals until the precipitate settles readily and leaves a clear supernatant liquid.

Filter and save the filtrate for the determination of magnesium.

Ignite the precipitate and weigh it as calcium oxide, 71.46 per cent of which is equivalent to Ca.

Magnesium

Use the filtrate from the separation of calcium. Acidify with hydrochloric acid.

Concentrate to about 100 c.c.

Add 20 c.c. of a saturated solution of sodium ammonium phosphate solution ($NH_4NaHPO_4.4H_2O$). Cool and make slightly but distinctly alkaline by adding ammonium hydroxide, drop by drop.

Allow the solution to stand four hours, then filter and wash with 3 per cent ammonium hydroxide.

Ignite the precipitate and weigh as magnesium pyrophosphate, 21.84 per cent of which is equivalent to magnesium. If manganese is present it is precipitated with the magnesium.

Correct for manganese after determining the quantity in a separate sample. The weight of manganese pyrophosphate is equivalent to 2.586 times the weight of manganese.

Calculation of Total Hardness.—Total hardness may be computed from the results of the determinations of calcium and magnesium in the sample. Iron and other metals must be included in the calculation if they are present in significant amounts. Total hardness as $CaCO_3$ is equal to $2.495 \times$ calcium $+ 4.115 \times$ magnesium.

Noncarbonate Hardness.—If the total hardness is greater than the carbonate and bicarbonate alkalinity, the amount of hardness equivalent to the alkalinity is called ''carbonate hardness.'' The amount of hardness in excess of this is called ''noncarbonate hardness.''

Residual Chlorine

Reagents.—

Orthotolidine Solution.—

Weigh 0.5 gm. of orthotolidine.

Grind to a thin paste in a mortar with 5 c.c. of dilute hydrochloric acid solution (below).

Add 150 to 200 c.c. distilled water. The orthotolidine should dissolve immediately.

Transfer to a liter graduate and dilute to 505 c.c. with distilled water. Add dilute hydrochloric acid to a final volume of 1000 c.c. Store in dark amber bottles for no longer than 6 months and do not expose to high or low temperatures.

Dilute Hydrochloric Acid.—

Add 150 c.c. concentrated hydrochloric acid, sp. gr. 1.18 to 1.19,
to 350 c.c. distilled water.

Sodium Hydroxide Solution, 4.5 N.—

Magnesium Sulphate Solution.—

Dissolve 20 gm. of magnesium sulphate in 100 c.c. distilled water. If necessary, eliminate any large chlorine demand in this and in the sodium hydroxide solution by treating to a slight residual with calcium or sodium hypochlorite solution.

Potassium Chromate-Dichromate Solution.—

Weak.—Dissolve 0.25 gm. of potassium dichromate
and 0.75 gm. of potassium chromate
in 1000 c.c. of distilled water.

Strong.—Dissolve the same quantities of chemicals in 100 c.c. of distilled water.

Boric Acid-Borax Buffer, pH 6.5.—

Dissolve 12.4 gm. boric acid in distilled water and dilute to 1000 c.c. with distilled water. This is approximately a 0.2 M solution and has a reaction of pH 4.6±.

Dissolve 3.8 gm. of borax ($Na_2B_4O_7$) in distilled water and dilute to 1000 c.c. with distilled water. This is a 0.01 M solution with a reaction of about pH 9.2.

Add sufficient borax solution, usually about 80 c.c., to a liter of the boric acid solution to prepare a buffer solution of pH 6.5.

Note.—The reagent used for detection and estimation of chlorine is not specific, but gives similar reactions with oxidizing agents and chlorine addition products. Exercise care, therefore, in interpreting the results. Nitrites, ferric compounds, manganic compounds, organic coloring matter, and chlorates are examples of interfering substances; these must be considered in evaluating the results. Turbidity interferes with the test. Do not confuse orthotolidine with orthotoluidine.

Technic.—

Place 100 c.c. of the sample in a 100 c.c. Nessler tube.

Warm to 20° C.

Add 2 c.c. of the orthotolidine solution, mix, and place in the dark for not less than 5 or more than 15 minutes.

Small amounts of chlorine give a yellow color. Larger amounts give an orange color. Compare the color with permanent standards, below. All tubes should match as to height of graduation mark.

Permanent Standards.—

Permanent standards for testing residual chlorine are available from many chemical supply houses. They may be made as follows:

Use 100 c.c. Nessler tubes. Place the proper volumes of dichromate-chromate solutions in the tube and dilute to the 100 c.c. mark with the boric acid-borax buffer.

CHLORINE STANDARDS, SCOTT FORMULA

CHLORINE PPM	C.C. WEAK DICHROMATE-CHROMATE	CHLORINE PPM	C.C. STRONG DICHROMATE-CHROMATE
0.01	0.4	0.50	2.6
0.02	0.8	0.70	3.6
0.03	1.3	1.00	5.2
0.05	2.2	1.50	8.0
0.07	3.1	2.00	11.2
0.10	4.4	3.00	18.0
0.15	7.4		
0.20	10.0		
0.25	13.0		
0.30	16.0		
0.40	21.0		

If the water contains over 1 part per million of iron or 0.01 part per million of manganic manganese, flocculate 200 c.c. with 2 c.c. of the magnesium sulphate solution and 2 c.c. of 4.5 N sodium hydroxide, clarify by centrifugalizing, and follow the standard procedure.

Interpretation of Chemical Analysis of Drinking Water

The following are ordinarily considered safe limits of impurities in drinking water of good quality from a *chemical* standpoint. Drinking water must never be considered safe, however, without a satisfactory *bacteriologic* analysis. The results below are expressed in *parts per million.*

IMPURITY	DESIRABLE	LIMIT
	Less than:	Not greater than:
Odor	None	Free from odor
Color	5	20
Turbidity	5	10
Ammonia nitrogen	0.04	0.9
Albuminoid nitrogen	0.05	0.1
Nitrite nitrogen	None	0.7
Nitrate nitrogen		0.5
Oxygen consuming power	1	2
Chlorides	50	250
Free residual chlorine	At least 0.2 (at tap)	Must not cause objectionable odor or taste
Total hardness (as $CaCO_3$)		250
		10.2
Noncarbonate hardness	Relative to total hardness	
Acidity	Free mineral acids must be absent	
Total solids	500	1000
Loss on ignition	Is a relative, not a precise, determination Represents not only organic matter but volatile decomposition products from carbonates, nitrates, and other components of the residue	
Alkalinity (as $CaCO_3$)	It is desirable to have the total alkalinity below 400 ppm; however, alkalinity is usually of no significance unless there is phenolphthalein alkalinity In the case of nontreated waters, the phenolphthalein alkalinity should not be greater than half of the total alkalinity In chemically treated waters, the phenolphthalein alkalinity (calculated as $CaCO_3$) should not be greater than 15 mg./L. plus 0.4 times the total alkalinity. In the case of waters softened by the lime soda process, the total alkalinity produced should not exceed the hardness by more than 35 ppm (calculated as $CaCO_3$)	
Minerals		
Silica		250
Magnesium	30	100
Calcium	Unimportant	250
Iron	0.1	0.3
Sulphates	100	500
Sodium and potassium	0	50
Aluminum		0.3
Manganese		0.5
Toxic Ions:		
Arsenic	None	0.05
Lead	None	0.1
Copper	None	3.0
Zinc	None	15.0
Fluorides	None	1.0
Selenium	None	0.05
Barium	None	None
Molecularly dehydrated phosphates (chem. treatment)	Less than 10.0	10.0

Bacteriologic Examination of Water from Swimming Pools and Bathing Places

For some time it has been the custom to disinfect swimming pools and bathing places with some form of chlorination. This method is highly efficient provided it is properly controlled. In order to test the efficiency of chlorination, bacteriologic examination of water from these pools is made daily in some places. It has been discovered that when water is taken from the pool and examined at once, a given bacteriologic result will be obtained which is quite different from that obtained when the water is examined several hours later. This is due to the fact that the residual chlorine present in the water continues to act after the water is taken from the pool; therefore, a sample examined later will show less pollution. The presence of even a minimum chlorine residue by chemical test shows the presence of germicidal chlorine. If germicidal chlorine is present, it necessarily follows that any bacteria present are going to be affected, and if a period of time elapses between sampling and testing, a reduction of the bacteria must result. If we have a sufficient period and a sufficient amount of residual chlorine, an otherwise polluted sample would be sterile when tested.

Mallmann and Schalm[1] demonstrated in a series of laboratory experiments that increasing alkalinities in water caused a decreased activity or delayed killing action of chlorine. It was also found that during periods of use, swimming pool water showed marked pollution as measured by colon bacilli and streptococci indices in the presence of residual chlorine contents of 0.2 to 0.5 p.p.m. and that during rest, the pollution evidenced during use disappeared, the rate of disappearance depending upon the type of treatment used.

Water taken at the pool-side and immediately examined will give an accurate picture of the bacterial content of said water. In order to examine water and get accurate results at a later period, it is necessary to treat the sample in such way that the residual chlorine will not continue to act upon the sample after it is collected.

Extended studies of Mallmann and Cary[2] and Fletcher and Link[3] have demonstrated that samples collected in sodium thiosulphate-treated bottles give an actual picture of the water at the time of the sample; therefore, this type of bottle should be used.

BACTERIOLOGIC EXAMINATION OF MILK

Macroscopic Colony Count—Petri Dish Method

Phosphate Buffered Distilled Water for Dilutions.—

Stock Solution of Phosphate Buffer.—

Dissolve 34 gm. of monobasic potassium phosphate (KH_2PO_4)
in 500 c.c. distilled water.
Adjust the reaction to pH 7.2 with N/1 sodium hydroxide.
Dilute to 1,000 c.c. with distilled water.

For Use.—

Add 1.25 c.c. of the stock phosphate buffer to distilled water, and dilute to 1,000 c.c. with distilled water.

Preparation of Bottles.—

Use resistant glass bottles, 4 ounce capacity, closed with rubber stoppers or preferably screw caps.

Place a mark at 99 c.c. with a diamond point pencil.

Fill with the phosphate buffered distilled water to the 99 c.c. mark, and sterilize in an autoclave at 15 pounds pressure for 30 minutes, after closing with the stopper. Do not use cotton plugs.

When cool, check for evaporation. Measure the quantity of fluid which must be added to make up the quantity to 99 c.c.

When preparing the bottles for use, add the 99 c.c. of buffered solution and the excess which is to be allowed for evaporation, before sterilizing.

If desired, prepare bottles marked also at 9 c.c.

Preparation of Dilutions of Milk.—

To make a 1:10 dilution, add 11 c.c. of the milk, in the manner described under Water Analysis, page 1661, to the bottle containing 99 c.c. of phosphate buffered distilled water. Replace cap and mix thoroughly.

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[1] Michigan Eng. Exper. Sta. Bull. 44, 1932.
[2] Am. Pub. Health A. 23: 35, 1933.
[3] Am. Pub. Health A. 23: 1255, 1933.

To make a 1:100 dilution, add 1 c.c. of distilled water to 99 c.c. of phosphate buffered distilled water. Mix as above.

Make a 1:1,000 dilution by transferring 1 c.c. from the 1:10 dilution to 99 c.c. of phosphate buffered distilled water.

To make a 1:10,000 dilution, transfer 1 c.c. from the 1:100 dilution to 99 c.c. of phosphate buffered distilled water.

In preparing the plates, measure 1 c.c. of the respective dilutions in sterile 1 c.c. pipettes, letting the column drain into the Petri dish and then blowing out the last drop. Do not rinse the pipette in the dilution waters.

Each plate is inoculated with 1 c.c. of its respective dilution of milk. Use milk agar M-111, melted and kept at a temperature of 45° C. Do not guess at the temperature, but keep the media in a regulated water bath, the thermometer being in one of the tubes (which will be discarded at the end of the test).

After solidifying, the plates are placed, upside down, in the incubator at 35° C. for forty-eight hours.

If, among the various dilutions, there are plates containing 30 to 300 colonies, these are selected for making the count.

The number of bacterial colonies on the plate is multiplied by the dilution of the milk in the plate for the number of bacteria in 1 c.c. of undiluted milk.

Several plates are counted. The average counts are taken.

If no plates show bacterial growth within these limits, the plates with bacteria numbering closest to 300 colonies are counted.

No plate that contains less than twenty colonies should be counted.

Counting should be done with a hand-lens, and all recognizable colonies included. In case any particles visible by this method are of doubtful nature, they should be examined with a compound microscope, to determine their true nature.

Microscopic Count of Bacteria (Breed Method)

Standardization of Microscope

A stage micrometer and mechanical stage are needed.

Place the stage micrometer on the stage of the microscope.

Use the oil immersion objective, and by adjusting the ocular and the draw tube, bring the diameter of the field to 0.16 mm. When so adjusted each field of the microscope covers an area of approximately 1/5000 sq. mm.

Record the number of the microscope, the number of the ocular, the objective used, and the length of the draw tube.

Counting the Bacteria

Cleanliness of glassware is more important than sterilization.

Slides must be thoroughly cleaned so that there is no fat present.

On a piece of white paper, rule off an area exactly 1 sq. cm.

Shake the sample of milk thoroughly.

Withdraw 0.01 c.c. in a 0.01 c.c. pipette.

Deposit on a clean slide.

Spread the drop uniformly over an area of 1 sq. cm. by means of a stiff needle. This is accomplished by placing the slide over the previously ruled square, and using it as a guide.

Dry the preparation in a warm place, upon a level surface, protected from dust. This drying should be accomplished within five to ten minutes. Excessive heat should be avoided, or the dry films will crack and peel from the slide in later handling.

Dip in xylol to remove fat. Usually one minute.

Drain, and dry.

Immerse in 90% alcohol for one or more minutes.

Transfer to a jar of Loeffler's methylene blue.

The slides are left in the stain until overstained.

Rinse in water and decolorize in alcohol.

This requires from several seconds to a minute or more. Observe the slide carefully during the process in order not to decolorize too long. When properly decolorized, the background of the film should show a faint blue tint. Poorly stained slides may be decolorized and restained without injury.

Examine the slides with the microscope adjusted so that the field covers an area of approximately 1/5000 sq. cm., as described above. The diameter of the field is 0.16 mm.

Each organism in a field represents 500,000 bacteria per c.c.

Count the number of bacteria in 10 fields.

Multiply the total by 50,000 to obtain the number of bacteria per c.c. of sample.

Count 100 fields when the number of bacteria is moderate, or when more accurate results are desired. Multiply the total in this case by 5,000 to obtain the number of bacteria in each c.c. of sample.

Tests for Control of Milk

Coliform Organisms

One of the most simple and effective methods for the control of pasteurized milk was devised by Mickle and Borman.[1] It is known as the ''Connecticut Three Point Program.'' This consists of the determination of the bacterial count by the Breed method (not more than 20,000 organisms in grade ''A'' milk, not more than 50,000 organisms in grade ''B'' milk); phosphatase determination (less than 0.05 mg. phenol liberated, see below); and the absence of colon bacilli in 0.1 c.c. of the milk.

The presence of colon bacilli in milk is tested by inoculating a series (3 to 5) of lauryl sulphate broth M-109 or brilliant green lactose bile broth M-108 with 0.01, 0.1, and 1.0 c.c. of milk.

Incubate all tubes for 48 hours at 37° C. and observe for gas formation. If the gas occupies more than 1/10 of the inverted vials in the test tubes containing the broth, streak on eosin-methylene blue agar M-72 from the broth and proceed as under ''Water Examination.''

Tests for Other Bacteria in Milk

Brucella.—

Centrifuge samples of milk.

Collect cream and sediment and mix both.

Inoculate tryptose agar plates M-16 or liver infusion agar plates M-26 with this mixture. Incubate in carbon dioxide atmosphere for a week.

Inoculate two guinea pigs with 5 c.c. of the cream-sediment mixture.

If the animals do not die in 7 weeks, sacrifice them and examine the liver and spleen for typical lesions. Inoculate plates with the material from these organs as above.

Testing Milk Sera for Brucellosis.—

This test may be carried out with milk from cows for the detection of those carrying *Brucella abortus* in the udders. Collect several samples of the fore milk from each quarter, using clean, sterilized vials of about one-half-ounce capacity. Before collecting the samples, place in each vial a small amount of rennin powder, about the amount that would go on the tip of a small knife blade. Draw the milk into the vial and place in a slanting position. The serum will separate out in about an hour at room temperature. Conduct the test with the milk sera in exactly the same manner as previously described for blood sera, p. 1573.

Typhoid and Other Salmonellae—

Use methods described under Water Examination, page 1663.

Tubercle Bacilli.—

The only reliable method is to centrifuge the milk; collect the cream and the sediment and inoculate guinea pigs with this mixture.

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[1] J. Milk Technol. 6: 231, 1943.

Streptococci.—

(a) **Hotis Test.—**

Add 9.5 c.c. of milk to 0.5 c.c. of a 0.5% sterile aqueous solution of brom cresol purple in sterile test tube.

Incubate overnight at 37° C.

Decolorization and curd formation indicates mastitis.

(b) **Plating.—**

Plate samples of milk on sodium azide blood plates M-21.

Carry out tests for further identification as described under "Streptococci," pages 1487 ff., and 1490 ff.

Acid-Producers, Molds, Yeasts.—

Use dextrose agar M-19 for their detection.

Brucella Abortus Ring Test

The ring test for diagnosis of brucellosis in dairy cattle was first described by Fleischhauer and Hermann[1] in 1937 and 1938. It is sufficiently sensitive to permit its use on pooled milk samples from individual herds, collected at the receiving platform in a dairy plant.[2] It has been found positive in pooled samples of milk where the milk from one infected animal had been mixed with that of as many as 5 to 15 noninfected or negative cows.

The present ring test is not a reliable method of diagnosing infection in individual animals, but it will identify approximately 80 per cent of the infected herds.

A number of factors may influence the results[3]: period of lactation, nonlactating infected animals, adult vaccination with strain 19 vaccine, method of collecting milk samples, souring and freezing of milk, amount of cream on the sample; and uniformity of antigens, procedures, and interpretation of results.

The antigen is deep blue. In milk from infected animals, the stained antigen agglutinates and collects on the fat droplets or cream, and as the cream rises, the antigen is carried with it. It therefore produces a deep blue cream line or ring when whole milk is tested. The skim milk fraction is white in strongly positive samples; in negative samples, the antigen remains and gives the skim milk fraction a light blue color, the cream ring being white.

The test is sometimes designated as A.B.R., meaning "*abortus* Bang ring" test.

Collection of Specimen.—

Approximately 3 c.c. of milk are collected from each of 2 or 3 milk cans of a producer, using a small long-handled dipper on the receiving line. For a dairy herd producing 5 or 6 cans of milk per day, this represents 2 such composite milk samples to be tested. For cream, approximately 5 c.c. are secured from each can on the receiving line, or taken from the vial containing the cream specimen normally collected by the creamery operator for butter-fat analysis.

Antigen.—

Obtain the parent antigen from the U. S. Bureau of Animal Industry, official *Brucella abortus plate antigen*, about 8% by volume; *or* the stock *tube antigen*, approximately 4% cells.

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[1] Berlin. tierärztl. Wchnschr. **53**: 527, 1937; **54**: 333, 1938.
[2] Roepke: Brucellosis, AAAS, 1950.
[3] Manthei: Brucellosis, AAAS, 1950.

Centrifuge the stock antigen in weighed centrifuge tubes.

Wash once by centrifugation with physiologic saline.

Discard the supernatant fluid and reweigh the tubes. This gives the weight of the bacterial paste.

Stir this paste thoroughly with a glass rod, adding a few drops of saline at a time, until a smooth thick creamy suspension of the cells is obtained in a small volume of saline.

The suspension of the cells must be stable at the low pH and high salt concentration of the staining solution used to stain the antigen. It must also be stable at pH 4.0, the pH of the wash fluid used to remove the excess dye, and the approximate pH of the final preparation.

The Staining Solution:

1. Dissolve 9 gm. of ammonium aluminum sulphate
   in 100 c.c. of distilled water.
   Add 30 c.c. of glycerin.
2. Dissolve 1 gm. hematoxylin
   in 2 c.c. of 85% ethyl alcohol by heating at 50° C.
   Add distilled water to a total volume of 100 c.c.
3. Mix the two solutions.

Add 0.17 gm. sodium iodate dissolved in 2 c.c. distilled water.

Wait 15 minutes for oxidation of the dye.

Dilute the dye 1:4.5 or 1:5 with 10% ammonium aluminum sulphate solution (a less deeply stained antigen may be made at this point if desired).

Allow to stand at room temperature for 12 to 20 hours.

Filter through a small filter paper to remove insoluble substances.

The final reaction should be pH 2.8 to 2.9.

This is the staining solution.

The Stained Antigen:

Add the concentrated suspension of washed cells to the staining solution in the proportion of 10 gm. of paste to 1,200 to 1,400 c.c. of the diluted stain.

Allow to stand at room temperature for 36 to 48 hours.

Collect the stained cells by centrifugation at 2,800 to 3,000 r.p.m. for 90 minutes.

Remove excess dye by suspending the stained cells in a wash fluid. (**Wash Fluid.**—This is composed of 0.4% sodium chloride and approximately 0.001 M lactic acid, the reaction adjusted to pH 3.8 to 4.0, using dilute sodium hydroxide solution.)

Centrifuge the suspension.

Repeat the washing process to a total of three washings, using a volume of wash fluid each time of approximately one-fourth to one-fifth the original volume of the staining solution.

Suspend the washed cells in a solution of 0.5% phenol in a mixture of equal parts of glycerin and physiologic saline, in a proportion of 1 gm. of the stained bacterial paste to 20 c.c. of the diluting fluid.

Adjust the final cell concentration to 4% cells by volume, as determined by the centrifuge method with the Fitch-Hopkins vaccine tube. In an emergency, a graduated centrifuge tube may be used.

Adjust to a final reaction of pH 3.8 to 4.0 preferably by means of the glass electrode method.

Technic of the Ring Test.—

(a) Milk:

Mix the specimens by gently inverting the collecting vials several times.

Pour sufficient milk, about 2 c.c., into a small test tube, 10 by 100 mm., to produce a column of milk approximately ¾ inch deep.

Add 2 drops of saturated solution of sodium bicarbonate to a test specimen, if it is poor quality milk, before adding the antigen.

Add the stained antigen to the milk in the proportion of 1 drop or 0.03 c.c. of antigen to each c.c. of milk.

Mix well for 1 minute by gently inverting the tube a number of times, in such manner as to thoroughly wash the antigen down the wall of the tube.

Examine the specimen after it has stood a few minutes, to check for adequate mixing. If not mixed enough, the process must be repeated. Avoid forming a foam.

Allow to stand at room temperature for 75 to 90 minutes, depending upon the temperature.

Results.—

–	cream line or ring that is white or nearly white; skim milk contains essentially all the stained antigen.
+	cream ring color approaches or equals that of the skim milk fraction.
++	cream ring color slightly greater that that of the skim milk fraction.
+++	cream ring significantly deeper blue than the skim milk layer.
++++	cream ring very dark blue and the skim milk fraction essentially white.

(b) Cream:

Place 1 c.c. of physiologic saline containing 1% sodium carbonate in a small tube (10 by 100 mm.) and add 12 drops of cream, using a dropper.

Add 1 drop of antigen.

Mix thoroughly.

Allow to stand at room temperature.

Read as in the milk test.

The Reductase Test[1]

(Methylene Blue Reduction Test of Milk)

The reductase test of milk is based on the fact that the color imparted to milk by a small quantity of methylene blue will disappear more or less quickly due to the activity of growing bacteria, presumably by their consumption of the oxygen dissolved in the milk.

The value of the methylene blue reduction test is the rapidity with which the quality of milk can be observed where laboratory facilities are limited.

Apparatus.—

10 c.c. pipettes.

1 c.c. pipettes with a small opening.

Test tubes 12 to 15 mm. in diameter.

Water-bath regulated at 37° C.

All glassware used should be sterilized in the dry heat sterilizer at 170° C. for two hours. Test tubes should be plugged with nonabsorbent cotton.

Reagent.—

Use standard tablets of methylene blue thiocyanate, certified by the Biological Stain Commission. These can be purchased from most biological supply houses.

Using sterile forceps, pick up one methylene blue tablet and place it in a flask containing 200 c.c., or slightly more, of boiling distilled water. When cool, the total quantity must be 200 c.c. Cool rapidly when the tablet has dissolved. The solution is not stable.

Technic.—

Place 1 c.c. of the methylene blue solution in a sterile test tube, using a buret.

Add 10 c.c. of the milk to be tested.

Insert sterile cork and invert gently. The color of the milk is now a robin's egg blue. The intensity of color will not be the same in all milk because of differences in milk fat dispersion.

Place the tubes in the water-bath at 37° C. and observe at stated intervals. The end point is reached when the blue color has disappeared and the milk has regained its normal color. In most cases, the color disappears uniformly throughout the entire mass of milk. If the color disappears in an uneven manner, the end point is taken as the time when the milk, after mixing, shows no blue color.

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[1] Abstracted from "Standard Methods of Milk Analysis," 6th Edition, 1934, published by the American Public Health Association.

Interpretation of Results.—

Class 1. *Good milk,* not decolorized in five and one-half hours.

Class 2. *Milk of fair average quality,* decolorized in less than five and one-half hours but not less than two hours.

Class 3. *Unsatisfactory milk,* decolorized in less than two hours but not in less than twenty minutes.

Class 4. *Very unsatisfactory milk,* decolorized in twenty minutes or less.

The Phosphatase Test for Pasteurization*

It is a matter of great importance for public health officials to know whether milk that is labeled "pasteurized" really has received sufficient heat treatment to make it safe. The phosphatase test is the first means the health officer has had to determine whether a sample of milk has been adequately heat treated.

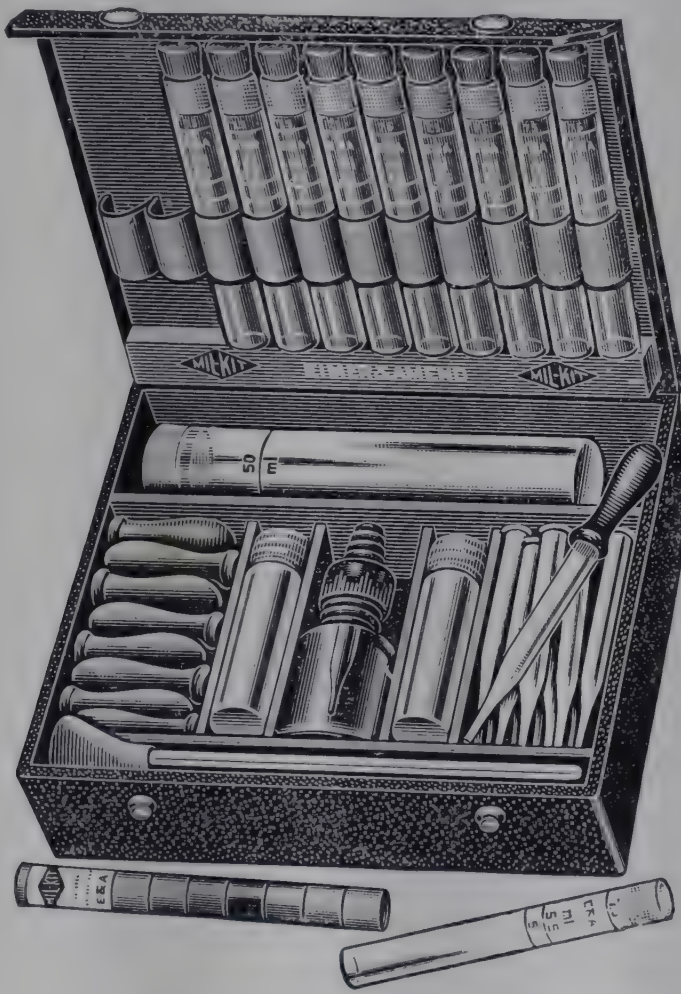

Fig. 405.—Milk kit, Eimer and Amend.

Numerous attempts have been made to cover this question in the past thirty years. The method devised by Rothenfusser[1] was designed to measure the effect of heat on the enzyme peroxidase. Various modifications of this test have been developed but were unsuccessful. Other tests for heated milk including the microscopic cellular test[2, 3] have failed to differentiate between pasteurized milk and milk undertreated in pasteurization.

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*The Mil-Kit by Eimer and Amend, New York, is recommended. It contains all the necessary equipment and is assembled in a portable leatherette case. It is especially adapted to field work or plant operation, as shown in Fig. 405. The reagents and chemicals for making these reagents may be procured from the Paul Lewis Laboratories, Inc., manufacturing chemists, 918-922 N. 4th St., Milwaukee, Wisconsin.

[1] Milchwirtschaftliches Zentr. 6: 468, 1910.

[2] J. A. M. A. 64: 821, 1915.

[3] J. Dairy Sc. 2: 189, 1919.

The phosphatase test is based upon the fact that this enzyme is always present in raw milk in abundance and is almost completely devitalized by the heat treatment required for the pasteurization of milk. The test was developed by Kay and Graham.[4] The method, as modified by Gilcreas and Davis,[5] is as follows:

Collection of Sample.—Proceed as directed in Methods of Analysis, A. O. A. C., *Standard Methods for the Examination of Dairy Products,* 1953, p. 258, except that no preservative shall be present and not more than 48 hours shall have elapsed between the time of sampling and receipt at the laboratory. If samples are refrigerated, observe precautions to prevent freezing.

The quantity of sample required depends upon the number of determinations to be made. For the usual analysis collect 250-500 c.c.; for the fat determination only, 5-60 c.c. will suffice.

In the case of bottled milk collect one or more bottles as prepared for sale. In sampling bulk milk, thoroughly mix by pouring from one clean vessel to another 3 or 4 times. If this procedure is impracticable, thoroughly stir the milk for at least 30 seconds with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk, continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified through the liquid.

Place the samples in nonabsorbent, air-tight containers and keep them in the cold, but at a temperature above freezing until ready for examination. When transported by mail, express, or otherwise, completely fill the containers, tightly stopper, and mark for identification. A suitable quantity of preservative (mercuric chloride, potassium dichromate, or formaldehyde) may be used unless the presence of the preservative is objectionable in connection with physical or chemical tests to be applied in addition to the determination of fat.

Reagents.—

(a) *Buffer Substrate.*—Dissolve 1.09 gm. of disodium phenyl phosphate[6] and 11.54 gm. of sodium Veronal in water saturated with chloroform and dilute to 1 liter. Add 10 c.c. chloroform per liter and store the reagent in a refrigerator.

(b) *Folin-Ciocalteu Phenol Reagent.*—Dissolve 100 gm. sodium tungstate, according to Folin, and 25 gm. sodium molybdate in 700 c.c. water in a 1500 c.c. flask connected by a ground-glass joint to a reflux condenser. Add 50 c.c. 85% phosphoric acid and 100 c.c. hydrochloric acid and reflux gently 10 hr. Cool, add 150 gm. lithium sulphate, 50 c.c. distilled water and 4 to 6 drops liquid bromine. Boil the mixture without a condenser 15 min. to remove excess bromine. Cool, transfer to a 1 liter flask, dilute to volume with water, and filter. (The finished reagent should have a golden yellow color; reject it if it has a greenish tint.) Keep the reagent in the refrigerator, protected from dust. For use dilute 1 volume of this stock reagent with 2 volumes of water.

(c) *Sodium Carbonate Solution.*—Prepare a 14% or 1.32 M solution of anhydrous sodium carbonate.

(d) *Filter Paper.*—Whatman No. 40, 11 cm. diameter. (Use only this grade as interfering substances are at times extracted from other grades.)

Permanent Phenol Standards.—

(a) *Color Solution, Gray.*—Dissolve in distilled water, 31.9 gm. of cobaltous chloride ($CoCl_2.6H_2O$), 67.5 gm. of cupric sulphate ($CuSO_4.5H_2O$), and 75 gm. of nickel sulphate ($NiSO_4.6H_2O$). Add 32 c.c. hydrochloric acid and 45 c.c. sulphuric acid and dilute to 500 c.c.

(b) *Color Solution, Red.*—Dissolve 476 gm. cobaltous chloride in distilled water and filter. To the filtrate add 100 c.c. hydrochloric acid and dilute to 1 liter.

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[4] J. Dairy Res. 6: 191, 1935.

[5] 25th Ann. Rpt. Intern. Assoc. Milk Sanitarians 5: 15, 1936; 11th Ann. Rpt., New York State Assoc. Dairy and Milk Inspection, 1937.

[6] The disodium phenyl phosphate *must* be absolutely free of phenol. No soap-containing phenol must be used in cleaning the glassware used in this test. Check the paraffin used on milk caps for traces of phenol.

(c) *Color Solution, Blue.*—Dissolve 300 gm. cupric sulphate in distilled water, add 20 c.c. sulphuric acid, and dilute to 1 liter. (Should crystals appear when solution is cooled to below 20°, warm slightly before using to insure complete solubility.)

Prepare permanent color standards equivalent to phenol concentrations of from 0.01 to 0.15 mg. per 0.5 c.c. of sample by combining the quantities of color solutions a, b, and c indicated in the table and diluting to 10 c.c. with distilled water in each case; e.g., 0.3 c.c. solution a + 0.106 c.c. solution b + 0.96 c.c. solution c + distilled water to make a volume of 10 c.c. is equivalent to a phenol concentration of 0.01 mg. in 0.5 c.c. of samples.

These color standards are suitable for use only in natural daylight. If, however, a turquoise blue, unglazed, opaque glass plate is used to deflect the light from a daylight lamp through the tubes of standards and sample, accurate color comparisons can be made in the absence of daylight. Since the standards are prepared for use only at 13 mm. depth of color, tubes of different diameter cannot be used for accurate work.

PREPARATION OF PERMANENT PHENOL STANDARDS

PHENOL	COLOR SOLUTIONS			
	GRAY (A)	RED (B)	BLUE (C)	H_2O (DISTILLED) (D)
mg. per 0.5 c.c. (μg/c.c.)	c.c.	c.c.	c.c.	c.c.
0.01 = 20	0.30	0.106	0.96	8.634
0.02 = 40	0.40	0.140	1.16	8.3
0.03 = 60	0.55	0.180	1.65	7.62
0.04 = 80	0.65	0.216	2.10	7.032
0.06 = 120	0.92	0.286	3.00	5.794
0.09 = 180	1.30	0.326	4.40	3.974
0.12 = 240	1.70	0.360	5.70	2.24
0.15 = 300	2.50	0.396	7.10	0.004

Determination.—Transfer 10 c.c. of the buffer substrate solution into a test tube 20 mm. x 160 mm. and add 0.5 c.c. milk to be tested. Add a few drops chloroform, mix thoroughly by rotating the tube, and cover to protect contents from dust. (Do not use rubber or cork stoppers; paper toweling placed over the open end of the tube is satisfactory.) Warm to 37-39° C. in a water-bath and incubate at 34-37° C. for not less than 18 and not more than 24 hours. After incubation add 4.5 c.c. of the diluted Folin-Ciocalteu reagent. Mix, and allow to stand for 3 minutes. Filter, and transfer 5 c.c. of the filtrate to a test tube of 13 mm. diameter. Add 1 c.c. of the sodium carbonate solution and mix thoroughly by rotating the tube. Place the tube in a boiling water-bath for 5 minutes and filter. Cool, and estimate the color of the filtrate by comparison with the permanent color standards in 13 mm. outside diameter tubes, or with photometer and 650 mμ filter.

Control Test.—

(To check deterioration of the reagents and the presence of interfering substances in the milk sample.)

To 10 c.c. of the buffer substrate solution, add 4.5 c.c. of the Folin-Ciocalteu reagent, and 0.5 c.c. of the milk sample. (Do not incubate.) Mix thoroughly, allow to stand 3 minutes, and filter. To 5 c.c. of the filtrate add 1 c.c. of the sodium carbonate solution, mix thoroughly by rotating the tube, heat in a boiling water-bath 5 minutes, and filter. Cool, and compare the color of the filtrate with the permanent color standards in 13 mm. outside diameter tubes, or with photometer and 650 mμ filter. If the phenol value obtained is greater than 20 μg subtract the excess from the phenol value of the incubated sample to obtain the phenol value indicative of pasteurization treatment.

A phenol value of 80 μg phenol per c.c. sample generally indicates milk heated to 143° F. for 30 minutes. A value greater than this indicates progressively inadequate heat treatment. In reporting results, give the μg of phenol per c.c. of sample as well as an interpretation as to whether the milk is pasteurized or underpasteurized.

False Positive Phosphatase Tests.—

In connection with the performance of phosphatase tests to determine whether or not milk has been properly pasteurized, attention must be called to an occasional ''false positive.'' Buck,[1] using the Gilcreas modification of the Kay and Graham technic, described above, has found in the past five years in the Baltimore Health Department examinations of pasteurized milk and ice cream from plants where the equipment and pasteurizing inspection showed everything in order, twenty-one instances of these so-called ''false positives.'' Leahy and others[2] have shown that certain strains of bacteria are capable of hydrolyzing the substrate used in the phosphatase test. Therefore, a word of caution must be uttered in interpretation of this test where great numbers of bacteria are present in milk. Buck found a certain thermophilic bacillus present in milk that would give such a false positive. This organism was isolated from the products of four Baltimore dairies. Buck suggested the name *Lactobacillus enzymothermophilus* for this organism. He found that this organism produced an enzyme which was destroyed by heating at 185° F. for one minute. He found that production of the bacterial phosphatase in milk at pasteurizing temperatures of 142° to 144° F. began after a 90-minute holding period. The factors governing the production of the bacterial phosphatase are the inoculum number, the temperature, and the time period.

False positives are due to bacterial phosphatase enzyme production from thermophilic organisms, and not to inefficiency in pasteurization.

CHEMICAL MILK ANALYSIS

Reagents

N/10 Sodium Hydroxide.—

To about 50 c.c. distilled water in a 100 c.c. volumetric flask
Add 10 c.c. N/1 sodium hydroxide solution.
Dilute to 100 c.c. with distilled water.

Benedict's Volumetric Solution.—

The method of preparing and titrating Benedict's volumetric solution will be found on page 54.

Bogg's Reagent for Protein.—

(1) Phosphotungstic acid	25 grams
Distilled water	125 c.c.
(2) Concentrated hydrochloric acid	25 c.c.
Distilled water	125 c.c.

When the phosphotungstic acid is completely dissolved, mix the two solutions. This reagent is quite stable if kept in a dark glass bottle.

1% Alcoholic Phenolphthalein.—Dissolve 1 gram phenolphthalein in 100 c.c. 95% alcohol.

Milk-Fat Mixture.—Equal parts of concentrated hydrochloric acid and amyl alcohol.*

75% Sulphuric Acid.—

Distilled water	25 c.c.
Sulphuric acid, c.p., conc.	75 c.c.

Caution: Add the sulphuric acid slowly to the distilled water in a Pyrex flask, cooling it under running tap water.

Reaction

Technic.—

Make a preliminary determination of the reaction with litmus paper. If the milk is alkaline, the degree of alkalinity is determined by titration with N/10 hydrochloric acid; if it is acid, the degree of acidity is determined by titration with N/10 sodium hydroxide solution. The reaction of milk is usually amphoteric; that is, it reacts red to blue litmus, and blue to red litmus. Frequently, however, it is alkaline.

Fill a buret with N/10 sodium hydroxide (if the milk is acid).

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*Make fresh each day.

[1] Am. J. Pub. Health 32: 1224, 1942.

[2] J. Milk Tech. 3: 183, 1940.

Place 10 c.c. of milk in a casserole.

Add about 50 c.c. distilled water, and 2 drops of 1% alcoholic phenolphthalein.

Add the sodium hydroxide drop by drop from the buret, stirring constantly, until the first permanent pink color is obtained. This is the end-point.

Read from the buret the number of c.c. of N/10 sodium hydroxide used. Multiply this number by 10 to obtain the degree of acidity.

Example.—If 1.5 c.c. of N/10 sodium hydroxide were used to neutralize 10 c.c. of milk, the degree of acidity is 10 × 1.5, or 15 degrees. The degree of acidity is equal to the number of c.c. of N/10 sodium hydroxide required to neutralize 100 c.c. milk An acidity of 15 to 25 degrees may be observed within six hours after milking; forty-eight hours after, the acidity is generally between 100 and 125. This applies more to cow's milk than to human milk.

Specific Gravity

Take the specific gravity with a urinometer. If a bacteriologic examination is to be made, enough milk is transferred to a sterile container for that purpose before the urinometer is admitted. Normal specific gravity is 1.028-1.032. The milk should be cooled to 60° F. before taking the specific gravity.

Color

The normal color of human milk is pale bluish white.

Lactose

Fill a buret, graduated in 0.1 c.c., with milk. Place 25 c.c. Benedict's volumetric solution in a casserole. Add 5-10 grams sodium carbonate, and a pinch of powdered pumice.

Bring the solution in the casserole to a boil and continue boiling until the end-point is reached.

Add the milk to the Benedict's solution in the casserole, a drop at a time, stirring after the addition of each drop. The milk should be added very slowly to the solution in the casserole, and some time allowed to pass before the addition of each drop, as reduction of the copper takes place very slowly.

Continue adding the milk, and boiling, until the blue color of the copper sulphate has entirely disappeared, and is replaced by a gray color. This is the end-point.

Read from the buret the number of c.c. of milk used. Twenty-five c.c. Benedict's volumetric solution are reduced by 67 mg. lactose.

The number of c.c. of milk that were used to bring about the reduction of the copper sulphate, therefore, contained 67 mg. of lactose. From this figure, the number of grams of lactose per 100 c.c. milk, or the percentage of lactose, can be computed according to the following example.

Example.—It required 4.0 c.c. of milk to reduce 25 c.c. Benedict's volumetric solution. 4.0 c.c. milk, therefore, contain 67 mg. lactose. 1 c.c. milk contains 67 divided by 4, or 16.75 mg. lactose. 100 c.c. milk contain 16.75 × 100, or 1675 mg. lactose. 1675 mg. = 1.675 grams (1675 ÷ 1000). The percentage of lactose in the milk is, therefore, 1.675.

Normal percentage of lactose in human milk is 6 to 7; in cow's milk, 4.3 to 7.

Fat

Leffmann-Beam Method

Use the modified Babcock tube, made for 5 c.c. quantities of milk. Place 5 c.c. milk in the Babcock tube.

Add 1 c.c. milk-fat mixture. Mix thoroughly by rotating.

Add 75% sulphuric acid to the mixture, mixing thoroughly as the acid is added, until the meniscus stands at the 0 mark on the stem. The solution becomes quite hot, and must be centrifuged before it cools.

Centrifuge immediately at 1,000 revolutions per minute for three minutes. The fat will be separated, and may be read in percentage on the stem. Each mark on the stem is 0.2% of fat. Read the percentage of fat from the stem and record. Read from the top of the meniscus.

Normal percentage of fat in human milk is 3 to 5; in cow's milk, it is 3.5 to 4.

Protein

Bogg's Modification of the Esbach Method

If human milk is to be examined, dilute, accurately, 1:10 with distilled water. If cow's milk is being tested, dilute 1:20 with distilled water. Place the milk in an Esbach tube until the bottom of the meniscus stands at the mark "U."

Fill to the mark "R" with Bogg's reagent.

Mix by inverting several times, taking care to prevent the formation of air bubbles.

Allow to stand in an upright position for 24 hours. Read from the graduation marks the number of grams of dried protein per 1,000 c.c., or the height of the coagulum. Divide this result by 10 to obtain the number of grams in 100 c.c. Multiply this by the dilution of the milk. This gives the number of grams of protein in 100 c.c., or the percentage.

Example: Human milk was used, and the dilution was 1:10. The reading on the Esbach tube is 1.25. 1.25 ÷ 10 gives 0.125, or the number of grams of protein in 100 c.c. diluted milk. 0.125 × 10 = 1.25 grams of protein in 100 c.c. milk, or 1.25% protein.

Normal protein content of human milk is 1.0 to 2.25%; of cow's milk is 1.5 to 4%.

Total Solids

Total solids are roughly calculated from the lactometer readings according to the following formula:

Let "L" equal the last two figures of the specific gravity.

Let "F" equal the percentage of fat found in the milk.

Total solids equals:

$$(L \div 4) + (0.2 \times F) + F.$$

Example.—Specific gravity was 1.028. Percentage of fat was 4. 28 ÷ 4 is 7; 0.2 × 4 (F) is 0.8; 7 + 0.8 + 4 = 11.8, the total solids.

Total Protein

Compute the total nitrogen by the method described for total nitrogen in urine (page 144). Report "total nitrogen found." Human milk contains 9% nonprotein nitrogen. Subtract 9 from the total nitrogen found, and multiply the result by 6.34, which will give the total protein present.

Formaldehyde in Milk

Place 5 c.c. sulphuric acid, c.p., concentrated, in a test tube.

Add to it 15 drops of 10% ferric chloride solution. Great caution must be taken in the addition of the ferric chloride solution to the sulphuric acid. Do not add it too rapidly, and avoid undue agitation of the contents. The proportion of ferric chloride is 3 drops to each c.c. of sulphuric acid.

Fill a large test tube to within about 1 inch of the top with the milk to be tested.

Allow 1 or 2 c.c. of the sulphuric acid and ferric chloride mixture to run slowly down the side of the tube so that it will collect on the bottom of the tube without mixing with the milk.

Allow to stand about 5 minutes.

The development of a permanganate ring at the point of contact indicates the presence of formaldehyde.

Care must be taken not to use too much reagent, which may obscure the reaction.

FOOD POISONING

The term "food poisoning" is a very broad one. Illnesses included under it are caused by a variety of factors in which bacteria play a very important part. A number of metallic compounds may produce poisoning. Certain foods are poisonous due to the presence of alkaloids or more obscure but related substances. Attention, too, must be called to the fact that food allergy may be responsible for certain cases of so-called food poisoning. Finally, there are certain outbreaks in which no definite causative factor can be found. Epidemiologic evidence and clinical symptoms may give us a clue, but at other times this information is of no assistance. In this chapter we shall concern ourselves only with the bacteriologic examinations of suspected food. There are three groups which must be investigated: (1) *Clostridium botulinum;* (2) certain members of the Salmonella group; and (3) Staphylococci. In a few instances members of the dysentery group and streptococci as well as Proteus have been accused of being responsible for these attacks. Recently *Clostridium perfingens* type *F*, which is nonhemolytic, has also been implicated.

The Koser[3] procedure, detailed here, is recommended. It is as follows:

Laboratory Procedure

A. First Examination.—

1. Prepare a stained smear (Gram stain) directly from the foodstuff, preferably the liquid portion.

2. With a loopful of the foodstuff,[4] streak in succession two agar plates, preferably a meat infusion agar M-15. With another loopful streak two plates of eosin-methylene blue agar M-71, 72. Incubate for eighteen to twenty-four hours at 37° C.

3. Inoculate a tube of broth, preferably prepared from meat infusion M-4, with a small amount of the sample. Incubate at 37° C.

4. In order to determine the presence of Staphylococcus, use the agar plate medium described above under (2) and follow this up by the usual test for identification of staphylococcus, such as coagulase test (R-4), etc. Also make stain to determine whether the coccus is gram-negative or gram-positive. Special Petri dish with special anaerobic cover (see special plate made by Baltimore Biological Company, page 1470) is recommended. This plate should contain thioglycollate agar M-49. Staphylococci associated with food poisoning are often found to be facultative anaerobic; that is, they will grow on anaerobic cultures and also on this special medium.

If the poisoning is suspected or definitely known to be botulism the following steps should be included:

5. Inoculate each of three tubes of chopped meat medium M-12 with approximately a gram or 1 c.c. of the sample.

Immediately after inoculation two of the three tubes should be heated to 80° C. for twenty minutes to destroy vegetative cells. If an anaerobic jar is not available, the three tubes should be layered with about 1 c.c. of sterile petrolatum to form a seal. Incubation should be 35° to 37° C.

6. A portion of the sample should also be used to test for the presence of toxin. Inject subcutaneously at least two guinea pigs or two white mice with a portion of the food. In most cases it will be necessary to centrifuge the sample before injection to eliminate gross contamination. Use 0.5 to 1.0 c.c. of the supernatant fluid for injection. Where abundant toxin formation is suspected, and a sufficiently large sample is available, it is desirable to feed portions to two guinea pigs.

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[3] Am. J. Pub. Health 24: 203, 1934.

[4] If the only sample submitted is an empty jar or can, the interior should be thoroughly washed out with a few c.c. of sterile salt solution or sterile broth. The washings can then be used for the examination.

Should death of any of the animals occur, controls with antitoxin of types A and B should be included. Inject subcutaneously two more animals with a protective dose of type A antitoxin (usually 1 c.c. is sufficient) and an additional two with type B antitoxin. These four animals should then be injected with the foodstuff in the same way as the original two which did not receive antitoxin. If the antitoxic sera are not at hand, these controls should be performed as soon as they are available.

B. Subsequent Examinations.—

1. Microscopic examination of the stained smear will give an idea of the relative abundance of bacteria in the product and of the morphologic types present.

2. Examine the eosin-methylene blue plates for the presence of colorless colonies. If suspicious looking colonies are found, fish several to Kligler medium M-79 or T.S.I. medium M-80. The identification of suspected intestinal pathogens requires additional procedure and is described on pages 1632 ff.

Examine also the nutrient agar plates and make Gram stains of several representative colonies. Note especially if Staphylococcus or Streptococcus colonies are present in considerable numbers. Proceed with the identification as recommended on pages 1485, 1487 ff.

3. The purpose of using a tube of broth is to detect by enrichment and subsequent plating any Salmonella, Staphylococci, or other types which might have been missed by direct plating. If the steps listed under A2 and B2 have yielded no information of value, streak eosin-methylene blue plates and nutrient agar plates from this broth culture. The examination of these plates should then proceed as given under item B2.

4 and 5. (If suspected botulism.) The condition of the animals which were injected under item A5 will afford preliminary evidence of the presence or absence of botulinum toxin in the food. This should now be supplemented by examination of the meat medium tubes inoculated under item A4.

These tubes should be incubated for at least three to four days. Note any macroscopic evidence of growth and prepare Gram stains from each tube. Note whether gram-positive bacilli, with or without subterminal spores, are present. Select one or more tubes for a toxicity test similar to that carried out with the original sample under item A5. The result of this will serve to confirm that secured with the food sample and at times it may be of great value in affording some additional information. In the event of a positive test the meat medium may be used for further purification and isolation of the culture, if this is desired.

C. Examination of Feces.—

Samples of excreta are often of value, particularly in those instances where the suspected foodstuff has been consumed or destroyed. If fecal specimens are taken, especial endeavor should be made to procure them during the acute stages of illness. It is believed that no detailed laboratory procedure need be given here, since the examination would follow the usual steps employed for isolation of organisms of the enteric group from feces (see pp. 1629 ff.).

D. Examination of Necropsy Material.—

If such material is available following acute gastrointestinal illness which might have been caused by Salmonella or dysentery organisms, cultures can be made from contents of the colon, spleen, and mesenteric lymph nodes. Positive cultures, which are definitely identified as Salmonella or dysentery types, may be of value in determining the cause of the illness. Positive cultures are usually not obtained from the blood, but where a definite Salmonella type has been isolated from the blood it is often of value when taken in connection with the other findings.

In cases of botulism, toxin can sometimes be demonstrated in blood or in bowel contents by animal injection along with the use of specific antitoxins. It is necessary to centrifuge or filter bowel contents to get rid of the numerous miscellaneous bacteria which are present.

Interpretation of Results

Since some outbreaks of food poisoning are due to other than bacteriologic causes, perhaps the first duty of the bacteriologist is to realize that his part of the examination may at times prove to be fruitless and that he should not feel obligated to attach blame to any organism which happens to be conspicuous.

The laboratory is often asked for proof that the actual organism obtained is in reality the cause of the trouble. Unfortunately, symptoms of typical acute gastrointestinal illness can rarely, if ever, be reproduced in the ordinary laboratory animals by feeding. Results obtained by the injection of suspected cultures are open to question and should not be advanced as sole proof of an etiologic role of the organism in question.

However, if a member of the Salmonella group has been isolated from the suspected food, one may rest assured that beyond reasonable doubt he has obtained the causative agent. This conclusion seems justified since these organisms are not ordinarily encountered in foodstuffs and since there is now good evidence that they are a definite cause of food poisoning. In the event that it has been possible to isolate a similar Salmonella type from specimens of feces or from necropsy material, additional confirmation is thereby obtained.

CAT TEST TO DETERMINE STAPHYLOCOCCIC FOOD POISONING

In the case of Staphylococcus findings in connection with food poisoning, it is considered that this food poisoning is due to the presence in the material of staphylococcus enterotoxin. Dolman and his associates[1] first described the kitten intra-abdominal test. This test consisted in injecting specially prepared culture filtrates into the abdominal cavity of very young kittens after inactivation of the lethal exotoxins by heat, Formalin, or neutralization by specific antitoxin. After a variable period of time, toxic filtrates containing the Staphylococci will produce vomiting and diarrhea in these kittens.

The method for preparation of the staphylococcus toxin has been described by Hammon and Favorite.*

Use trypticase soy agar M-18 in Petri dishes or Kolle flasks. Inoculate plates and incubate at 37° C. for 40 hours in 30% carbon dioxide. Put plates in an anaerobic jar, evacuate the air, and introduce carbon dioxide 30%. Squeeze the culture through cheese cloth, centrifuge at high speed, decant the supernatant fluid, and put through a Seitz filter. This is used to inoculate cats.

Chapman[2] suggested a rapid presumptive test for the examination of foods suspected of having caused food poisoning.

The food is triturated with a small amount of sterile broth M-1. To 0.5 c.c. of the filtrate of this extract add 0.5 c.c. citrated or oxalated human plasma. Incubate and observe for coagulation.

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*J. Bact. 41: 305, 1941.

[1] Canad. Pub. Health J. 27: 489, 1936.

[2] Food Research 9: 377, 1944.

A similar shortened method to be used with Staphylococcus colonies was described by Lominski and Grossfeld.[3] These authors suggested the addition of 2 to 3 loopfuls of the material to 0.5 c.c. of citrated or oxalated plasma which was diluted 4 to 8 times with saline. The mixture is incubated at 37° C. and read after ½ hour, 1, 6, and 12 hours. This method permits the saving of plasma.

Cat Technic: Place the cat, without anesthesia, on its back on animal board and shave the inner aspect of one thigh to a point just below the knee. Slight pressure over the vein near the inguinal region enables one easily to see and enter the saphenous vein at about the level of the knee, or just above, with a sharp 26 gauge needle. After releasing the pressure above, grasp the syringe and leg together with one hand and make the injection slowly. Depending on the toxin, from 0.5 c.c. to 5 c.c. may be required. The toxin must first be heated for thirty minutes in a boiling water bath. The precipitate should be sedimented and the supernatant liquid used as inoculum. When heat is used, the supernatant liquid from well-centrifuged cultures may be safely used without filtration. Sterility, in practice, appears to be unnecessary.

The train of **symptoms** observed after an intravenous inoculation of enterotoxin is as follows: In from 15 minutes to 2 hours, most frequently in about 30 minutes, vomiting occurs, preceded by nausea. Coarse tremors are usually noted and the hair stands erect. This is interpreted as a chill because the temperature rises, sometimes to 106°, after 2 to 4 hours.

Diarrhea is conspicuous but may be mild. It persists for several hours. Vomiting may recur at intervals of from 5 minutes to 1 hour over a period of 2 to 3 hours. After 3 to 4 hours, the cat is noticed to be less ill, and 24 to 48 hours later it appears to be normal. Since vomiting and occasionally diarrhea are symptoms of the very common and highly infectious type of feline epizoötic panleukopenia, it is quite important to use cats known to be in good health. A moderate-sized meal eaten shortly before the inoculation of enterotoxin has been found to increase the effectiveness of the vomiting stimulus, and noted refusal of the offered meal aids distinctly in the elimination of sick animals.

In the case of botulism there is fortunately direct evidence in the injection and feeding of animals and the protection afforded by a specific antitoxic serum.

TABLE 164.—DIFFERENCES BETWEEN BOTULISM AND FOOD INFECTIONS

COMPILED BY BURKE AND MAY*

	BOTULISM	FOOD INFECTIONS
Cause	Botulinus toxin	Salmonellae
Fever	Not characteristic; temperature usually subnormal	Characteristic; acute
Occurring	Mainly in winter	Mainly in summer
Associated with	Preserved foods	Fresh foods, or freshly contaminated foods, usually meat or milk
Condition of bowels	Constipation, rarely diarrhea	Diarrhea
Visual disturbances	Double vision; ptosis of lids	Absent
Abdominal pain	Absent	Present
Onset	Usually gradual	Sudden
Incubation period	Variable, usually from twelve hours to several days	Short, usually from six to twelve hours
Throat	Swallowing difficult	Normal
Treatment	Antitoxin	Systemic
Mortality	From 60 to 70%	From 1 to 2%

*From Stitt, E. R.: Practical Bacteriology, Blood Work and Parasitology, P. Blakiston's Son & Co.

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[3] Brit. M. J. 1: 854, 1944.

There is considerable difference of opinion as to the different media and methods which should be employed; the outline, page 1687, however, is serviceable.

SPECIAL TESTS IN CONNECTION WITH EXAMINATION OF FOOD CONTAMINATION BY CHEMICALS AND SPOILAGE*

There are a number of tests advocated for the control of products coming within the scope of health department activities. These products are spoilage of certain fragile food materials, cyanide in metal polish, etc.

Korff and Kaplan[1] have been particularly active in developing testing outfits and providing their inspectors with simple and proper equipment for gathering material for making these tests. With their permission, the method and equipment used by Baltimore City Health Department to make certain analytical examinations in the field are here presented.

Field Equipment.—Each inspector is equipped with a leather case containing the usual detention cards, violation notices, copies of laws, educational forms and leaflets, sample identification cards, and such conventional equipment as flashlights, thermometer, can opener, and meat trier. They have paper bags and paper spoons for collecting food samples; sterile 4 oz. bottles for water samples, and sampling vials for food utensil swabbing. They are also supplied with portable apparatus for detecting fluorides, cyanides, cadmium, arsenic, sulphites; also equipment for testing strength of chlorine solutions used as disinfectants and for the detection of spoilage of shucked oysters and crab meat. Where possible, reagents are kept in uniform glass vials, each case being fitted with a wooden rack to hold the apparatus. All solutions, test papers, and other equipment are periodically replaced and reconditioned by the Bureau of Laboratories.

Analytical Procedures

1. Detection of Spoilage of Shucked Oysters.—According to Korff and Kaplan, pH measurements of oyster liquor is a useful adjunct to organoleptic examination in the detection of spoilage of shucked oysters. This has been demonstrated by several investigators.[2, 3] The pH of the fresh oysters varies between 6.0 and 7.0. Hydrogen ion concentrations between pH 5.4 and 5.8 are suspicious, with decomposition indicated when the pH values lie below this range.

Technic of the Field Examination for Spoilage of Shucked Oysters.—

Transfer 5 c.c. of oyster liquor to a test tube graduated at 5 c.c.

Add 0.5 c.c. of methyl red indicator solution (0.02% methyl red in 50% alcohol).

Mix. Compare the color with the methyl red color standards. These color standards range from pH 5.4 to 6.0 and are kept in sealed ampules. If the oysters appear to have been recently washed, place several drops of the indicator solution directly on the oyster. Production of a red color which persists indicates spoilage.

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*This information is given here with the permission of the Authors and the American Journal of Public Health.

[1] Korff, Ferdinand A., and Kaplan, Emanuel: Director, Bureau of Food Control, and Chief, Division of Chemistry, respectively, Bureau of Laboratories, Baltimore City Health Department, Baltimore, Md., Am. J. Pub. Health 32: 1110, 1942.

[2] Hunter, A. C., and Harrison, C. W.: Bacteriology and Chemistry of Oysters With Special Reference to Regulatory Control of Production, Handling and Shipment, Tech. Bull. 64: 60, 1928, U. S. Department of Agriculture.

[3] Baldwin, W. H., Puncochar, J. F., and Pottinger, S. R.: Fishery Market News 3: 3, 1941, U. S. Department of the Interior, Fish and Wildlife Service.

2. Detection of Spoilage of Crab Meat.—Decomposition of crab meat involves a progressive proteolysis, with an accompanying rapid rise in the ammonia content of the meat. The Nessler ammonia test as a means of differentiating fresh from spoiled crab meat was suggested in 1932 by Harris.[4]

Technic of Test Made by Inspectors.—

Transfer about 1 gm. of crab meat to a test tube.

Add several c.c. of distilled water and shake thoroughly.

Add 2 or 3 drops of Nessler's reagent (see page 244).

Spoilage of meat is indicated if a deep yellow or brown color develops immediately. This test is used only as an aid to organoleptic examination of the suspected crab meat.

3. Detection of Cyanide in Metal Polish.—Williams, in 1930,[5] pointed out the possible role of cyanide-containing metal polish in cases of gastroenteritis. The Maryland State Department of Health in 1938 adopted regulations governing retail sale or use of cyanide-containing preparations. Many other communities have similar legislation.[5]

Sodium picrate test papers[6] are used conveniently for qualitative detection of cyanide in metal polishes in the field. Moisten the test paper with water, then suspend it in the container of suspected polish. Take care that the paper does not come in contact with the material. If the concentration of cyanide (as KCN) exceeds 0.5 per cent, the paper will turn orange and then brick red in 5 to 10 minutes. This reaction is not wholly specific for cyanide; nevertheless, it serves as a ready screening test in the field.

4. Cadmium.—There has been a tendency on the part of equipment manufacturing companies to use cadmium for plating utensils and equipment. This was brought about because of difficulty in obtaining aluminum and stainless steel. A number of food poisoning outbreaks attributed to consumption of acid foods stored in cadmium-plated vessels have been reported.[7] Warnings of the dangers of the use of cadmium-lined utensils have been issued to the newspapers by the Federal Security Agency.

Modified Field Test for Cadmium.*—

Reagents.—

1 oz. dropping bottle containing 10% nitric acid.

1 oz. dropping bottle containing 40% sodium sulphide.

Technic.—

Place 1 drop of 10% nitric acid on the surface to be tested.

Wait 5 to 10 seconds, then add a drop of 40% sodium sulphide solution to the drop of acid on the metal. A bright yellow color indicates the presence of cadmium.

If the surface is such that it is not feasible to use the liquids directly, dip a cotton swab into the nitric acid, and rub over a small area for about 5 to 10 seconds. Then place a drop of the sodium sulphide reagent on the swab. Instantaneous appearance of a canary yellow color on the swab indicates presence of cadmium.

Note: Reagents should be renewed at least once a month.

5. Available Chlorine in Disinfectant Solutions.—Regulations of the Maryland State Board of Health require that all food containers (drinking glasses, plates, forks, etc.) used in establishments dispensing food or drink, after cleaning and rinsing, must be disinfected either by immersing in water at 180° F. or above, or by immersion in a solution containing not less than 100 p.p.m. of available chlorine.

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*This is a modification of the method outlined in this article, and is a personal communication to the author (From Korff).

[4] Am. J. Hyg. 15: 260-275, 1932.

[5] J. A. M. A. 94: 627-630, 1930.

[6] Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, ed. 5, 1940, p. 366.

[7] Cadmium Poisoning, Pub. Health Rep. 17: 601, 1942.

Strength of chlorine rinse waters is determined by a modified orthotolidine test.[8] Inspectors carry in their kit a 2 oz. dropping bottle of orthotolidine solution, a 100 p.p.m. permanent chlorine standard in a sealed ampule, a test tube of the same diameter as the standard ampule and graduated at 10 c.c. and a medicine dropper calibrated to deliver 20 drops of water per c.c.

Technic.—

(*a*) Add 10 c.c. of tap water to the tube.
Follow by 4 drops (0.2 c.c.) of the rinse water being tested.
Add 1 c.c. of the orthotolidine solution.

Wait 5 to 10 minutes, then compare with the standard. The permanent standard actually corresponds to 2 p.p.m. of chlorine. The sample is diluted 1 to 50 in the test (0.2 to 10). Permanent standards are prepared by the method of Scott,[9] which permits color matching irrespective of the tube length.

(b) A compact portable iodimetric testing outfit for residual chlorine has been prepared for dairy inspectors. This consists of Barnes dropping vials containing potassium iodide-starch solution, lactic acid solution, and standard sodium thiosulphate solution. Transfer the rinse water to a vial graduated at 10 c.c. Adjust the standard solution so that one drop is equivalent to 20 p.p.m. of available chlorine when 10 c.c. of rinse water is titrated.

This iodimetric method gives a more exact evaluation of the strength of chlorine rinse water than the orthotolidine test. It may be used with chloramine preparations, whereas the orthotolidine test does not function well in the presence of chloramine compounds.

6. Fluoride-Containing Insecticide Powders.—Accidental poisoning from white sodium fluoride is not uncommon. The Maryland State Board of Health, in 1941, adopted regulations requiring insecticides containing sodium fluoride to be colored Nile blue as a warning. These regulations were adopted because of the widespread use of insecticides containing uncolored sodium fluoride in hotel, restaurant, and hospital kitchens in Maryland.[10]

Fluoride test paper is used. In the presence of strong acids, soluble fluorides decolorize the zirconium lake of sodium alizarin sulphonate.

Impregnate strips of filter paper with fluoride reagent.[11] Dry.

Moisten a test paper with dilute hydrochloric acid (the orthotolidine solution carried by the inspectors for the residual chlorine test may be used).

Touch the moist strip to the suspected powder.

In the presence of a large amount of soluble fluoride, decolorization of the paper will occur in about 5 seconds. Decolorization is evident in about 30 seconds when a 1 to 1,000 dilution of sodium fluoride powder in flour is used. Allow a reaction time of 5 minutes for traces of fluoride. Phosphates and oxalates may yield false tests. Positive findings, therefore, should be confirmed in the laboratory.

7. Detection of Sulphites in Meat Products.—Sulphites in ground meat serve partly as a preservative, but are used chiefly to deodorize and to restore the bright red color of fresh meat.[12] The A.O.A.C. qualitative test for sulphurous acid[13] has been adapted as a field test. Sulphite detection depends upon the evolution of hydrogen sulphide when material containing sulphide is treated with zinc and dilute mineral acid. The evolved hydrogen sulphide blackens a cotton dental roll moistened with lead acetate and contained in a Gutzeit scrubber tube.[14]

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[8] Standard Methods for the Examination of Water and Sewage, ed. 8, 1936, p. 20. American Public Health Association, New York, N. Y.

[9] Scott, R. D.: Water Works & Sewerage **82**: 399, 1935.

[10] Baltimore Health News **18**: 153-154, 1941.

[11] Snell, F. D., and Snell, C. T.: Colorimetric Methods of Analysis, 1936, p. 581, Van Nostrand.

Technic.—

Transfer about 10 gm. of ground meat to a wide mouth bottle containing 30 c.c. of 10 per cent sulphuric acid.

Stopper the bottle and shake moderately for 10 seconds. Allow several minutes for foam to subside.

Add 3 gm. of 30 mesh granulated zinc (arsenic- and sulphur-free).

Insert immediately a stopper containing a Gutzeit scrubber tube[14] previously fitted with a No. 2 cotton dental roll impregnated with 20 per cent lead acetate solution.

The cotton roll will become black in 5 to 10 minutes. After about 10 minutes, open the bottle. There will be a decided odor of hydrogen sulphide if sulphites are present. The test will detect as little as one grain of sodium sulphite in a pound of ground meat. Traces of sulphides in foods may yield false positive tests. Because of this, field outfits are intended for screening tests only. All samples which show positive reactions are submitted to the laboratory for confirmatory analyses.

8. **Arsenic Spray Residue.**—Presence of excessive amounts of spray residue on fruits and vegetables is suspected when there are unusual deposits on the foods. In order to eliminate the detention of truck, boat, or railroad shipments pending laboratory examination, testing for arsenic is done in the field. The test used is an adaptation of the official Gutzeit method for arsenic of the A.O.A.C. Similar modifications have been used by others.[15]

Use the equipment designed for the sulphite determination in ground meat. Fit a piece of glass tubing to the Gutzeit scrubber tube to hold a strip of sensitized mercuric bromide paper.

In addition, add 15 c.c. of 40 per cent stannous chloride solution to each liter of 10 per cent sulphuric acid reagent. In practice the acid used for the sulphite test contains the stannous chloride addition and is thus also suitable for the arsenic examination.

Directions are essentially the same as in the case of the sulphite examination. Introduce scrapings of suspected material into the generator. Add the acid and zinc. Allow the reaction to proceed for 30 minutes. A decided yellow to dark brown coloration of the sensitized mercuric bromide paper indicates the presence of arsenic.

9. **Food Utensil Swabbing.**—The Baltimore City Health Department food inspectors use the improved vials described by Buck[16] in 1941 to secure bacteriologic samples from drinking glasses and food utensils. These vials have a plastic screw type closure which holds a wooden applicator with cotton attached.

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[12] Leach, A. E., and Winton, A. L.: Food Inspection and Analysis, ed. 4, 1920, p. 216, Wiley.

[13] Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, ed. 5, 1940, p. 463.

[14] Ibid. p. 390.

[15] Mayo, N., and Taylor, J. J.: Florida Quart. Bull. 42, 2: 12, 1933.

[16] Buck, T. C.: Baltimore Health News 18: No. 7 (July), 1941.

Sterilize salt solution or broth directly in the vials. Numbers of vials may be carried by the inspectors in their carrying cases without danger of contaminating them. For more complete information refer to pages 1654 ff.

10. Other Tests.—Other equipment may be added to the inspectors' kits as needed. The Scharer modification of the phosphatase test for the detection of proper pasteurization of milk and cream[17] is adaptable to field use. Inspectors in soft drink bottling plants may use an outfit for testing alkalinity of wash waters. The foam test[18] for renovated butter and oleomargarine may be carried out in the field by means of a spoon and an alcohol lamp.

Discussion.—More than 6,000 field tests were made by the staff of the Bureau of Food Control of the Baltimore City Health Department during the years 1941 and 1942. The majority of these tests were examinations of chlorine rinse waters and food utensil swabbings. Other tests revealed that metal polish containing cyanide was in use in more than 200 food establishments, which led to regulations prohibiting its use. Field testing resulted in the condemnation of at least 50 lots of incipiently decomposed shucked oysters which could not have been readily detected by organoleptic means. Sulphite in ground meat is now rarely encountered, largely because meat dealers know that the Health Department has ready means of its detection.

The field test is never used as a basis for prosecution. The purpose and the intention of the field tests are to assist the inspectors' organoleptic senses; they are not meant to supplant the more critical examination of foods by trained chemists. Inspectors are cautioned to use the field methods for information and screening purposes only and to submit samples to the Bureau of Laboratories for confirmatory analyses.

EXAMINATION OF FLOUR FOR "ROPE" SPORE CONTENT

Method:[19]

Preparation of Special Broth:

Peptone	10 gm.
Beef extract	5.4 gm.
Sodium chloride	9 gm.
Distilled water	1 liter

pH = 7.2-7.3

Place in 5 c.c. quantities in $5/8$ inch diameter test tubes. Steam for 10 minutes on three successive days.

Sterilize bottles, pipettes, and measuring cylinder by heating at 150° C. for two hours.

Sterilize sodium chloride solution (0.5%) by boiling in a plugged flask for an hour.

Measure 400 c.c. of the sodium chloride solution into a sterile 16 ounce glass-stoppered bottle, and 100 c.c. into a sterile 8 ounce glass-stoppered bottle.

Heat 20 grams of purified silver sand to redness in a platinum capsule for some minutes and then pour, while hot, into the liquid in the 16 ounce bottle.

Weigh 20 grams of the flour on a sterile watch glass and transfer by means of sterile paper to the 16 ounce bottle. Then shake this bottle vigorously for two minutes and immediately transfer 1 and 2 c.c. quantities of the suspension to 5 c.c. tubes of broth.

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[17] Scharer, H.: J. Dairy Sci. **21**: 21-34, 1938.

[18] Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, ed. 5, 1940, p. 300.

[19] Amos, A. J., and Kent-Jones, D. W.: The Analyst **56**: 572, 1931.

Reshake the bottle and add 20 c.c. of the suspension at once to the liquid in the 8 ounce bottle; then shake it well. Transfer volumes of 4, 3, 2.5, 2, 1.7, 1.5, 1.3, 1.2, and 1 c.c. respectively of this dilution to 5 c.c. tubes of broth.

Thoroughly mix the contents of each tube, taken individually, and place the tube in a bath of boiling water, for 20 minutes. The water level must be above the level of the liquid in the tubes. Then thoroughly mix the contents of the tubes and place them in an incubator at 37° C. for 48 hours. Contents of tubes must be well mixed several times during the first 24 hours of incubation. After 48 hours, report the tube as positive or negative, according to whether or not the contained broth exhibits a pellicle. The number of rope spores (*Bacillus mesentericus)* is taken as the reciprocal of the smallest fraction of a gram of flour giving a positive test.

2 c.c. of primary suspension = 1/10 gram flour
1 c.c. of primary suspension = 1/20 gram flour
4 c.c. of 20:100 dilution = 1/30 gram flour
3 c.c. of 20:100 dilution = 1/40 gram flour
2.5 c.c. of 20:100 dilution = 1/48 gram flour
2 c.c. of 20:100 dilution = 1/60 gram flour
1.7 c.c. of 20:100 dilution = 1/70 gram flour
1.5 c.c. of 20:100 dilution = 1/80 gram flour
1.3 c.c. of 20:100 dilution = 1/92 gram flour
1.2 c.c. of 20:100 dilution = 1/100 gram flour
1 c.c. of 20:100 dilution = 1/120 gram flour

B. subtilis, another organism sometimes found in flour, forms a pellicle on the broth. This organism will not cause ropy bread. If the spore count is high, determine whether the pellicles are due to *subtilis* or *mesentericus,* by transplanting to agar slants.

B. mesentericus vulgatus forms a grayish-white growth which is at first moist and ''blister''-like in appearance, but later becomes dry and finely wrinkled.

B. subtilis gives a whitish, spreading growth, the edges of which appear as an interwoven mass of fine hairs.

The rope spore content of flour, unless particularly high, is of little significance in outbreaks of rope in commercial practice. The systems of fermentation, baking, and bread cooling employed by the baker are of greater significance.

When ropiness occurs, the following rules should be observed in fermenting the doughs:

(1) Avoid a long system of fermentation.
(2) Use a liberal quantity of yeast.
(3) Avoid low temperatures in the dough.
(4) Avoid slack doughs.

The dough should be well baked, and cooled as rapidly as possible in a well-ventilated place.

It is desirable to use flour of low rope spore content.

For the examination of thermophilic organisms in flour, and also in other foods, use dextrose agar M-19. It must be incubated at higher temperatures (45° and 50° C.).

EXAMINATION OF CANNED FOOD, DRESSINGS, NEEDLES, ETC., FOR STERILITY

Simple Rapid Test

The examination for sterility is made by placing a portion of the material to be tested in four fermentation tubes of chopped meat medium M-12. One is to be incubated aerobically at room temperature, one aerobically at 37° C., one anaerobically at room temperature, and one anaerobically at 37° C. The material from the canned food is obtained in the following manner:

Place about 5 c.c. of alcohol on the top of the can. Ignite. Flame the can opener to free it of bacteria. Cut a hole in the top of the can with the sterilized can opener. Using a piece of glass tubing which has been previously sterilized, remove a portion of the contents of the can, and using all sterile precautions, place in 4 tubes of chopped meat medium M-12. Two tubes are incubated at room temperature, the other two at 37° in the incubator. Both aerobic and anaerobic cultures should be made.

For dressings, use sterile scissors and sterile forceps to remove a portion of the material. Select a part of the material from the center of the package.

Flame the mouth of the fermentation tube, and drop the material into the broth, taking care not to touch the glass of the tube.

Needles, etc., are examined in the same manner as surgical dressings. For the examination of catgut, see below.

If no growth appears after ten days' incubation the material examined was sterile.

If a growth is obtained before ten days' incubation, make an examination to determine the type of bacteria present.

No report that the material was sterile may be given under ten days' incubation. A growth may be reported at once.

Evidence of growth is a cloudy medium. Smears may be made to confirm the evidence. Proceed in the usual manner to identify the bacteria.

Test for Sterility of Catgut

(Frank L. Meleney, M.D., and Mabel Chatfield, New York.)[1]

Run at least two tests on each sample; one aerobic, the other anaerobic. Seize the catgut from the container with a pair of long "alligator" forceps previously dipped into alcohol and flamed.

Transfer with sterile precautions to a test tube containing 40 c.c. sterile distilled water.

Place this tube in the incubator for twenty-four hours.

Transfer the catgut to a tube containing 1% sodium thiosulphate and 1% sodium carbonate solutions. Incubate for twenty-four hours.

Transfer to fermentation tubes containing thioglycollate broth M-48.

Incubate both cultures fifteen days before reporting sterile.

Tests should be made on the medium (1) for sterility, and (2) for growth-producing ability.

Tubes of the medium should be incubated, uninoculated, along with the sterility tests on the catgut.

Test organisms, such as anthrax and tetanus bacilli, should be planted in tubes of broth and incubated during the fifteen-day period.

Tests for Determination of Sterility of Solids, Such as Gauze, Surgical Dressing, and Related Products*

Method of Choice

Broth for Sterility Tests Under Aerobiosis.—

Use formula M-4 to which glucose has been added in 1 per cent concentration.

Do not use culture medium which has been kept longer than two weeks at room temperature or four weeks under refrigeration.

Broth for Sterility Tests Under Anaerobiosis.—

Litmus solution	10 c.c.
Gelatin	20 gm.
Broth for sterility tests under aerobiosis, a sufficient quantity to make	1,000 c.c.

The broth prepared for aerobiosis need not be filtered or sterilized before the addition of the other ingredients. Dissolve the gelatin in the broth by the aid of gentle heat, add the litmus test solution, and adjust the preliminary reaction to pH 7.6. Then, if necessary, add the albumen of two fresh eggs or its equivalent in desiccated egg albumen, heat to coagulate, and add distilled water to make 1,000 c.c.; filter until clear and distribute the filtrate in quantities of 40 c.c. each, as described under the broth for testing aerobic organisms. Sterilize in an autoclave at from 15 to 20 pounds pressure (121° to 125° C.) for from twenty to thirty minutes, or by any other suitable method. The final reaction should be between pH 7.2 and pH 7.4.

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*This material is taken from The Second Supplement to the Pharmacopoeia of the United States of America, Eleventh Decennial Revision (U. S. P. XI-1939 Supplement). Proper acknowledgment of this is seen on the reverse side of the title page of this book.

[1] Surg. Gynec. & Obst. 52: 430, 1931.

The 10 c.c. of litmus test solutions may be replaced by 0.2 gm. of azolitmin if preferred.

Do not use culture medium which has been kept longer than two weeks at room temperature or four weeks under refrigeration.

Honey Medium for Molds and Yeasts.—

Peptone	10 gm.
Honey	60 c.c.
Distilled water, a sufficient quantity to make	1,000 c.c.

Dissolve the peptone in the distilled water with gentle heat, add the honey, and adjust the preliminary reaction to pH 6.0. Filter if necessary and sterilize in an autoclave at from 15 to 20 pounds pressure (121° to 125° C.) for from twenty to thirty minutes or by any other suitable method. The final reaction should approximate pH 5.6.

Sterilized Distilled Water.—

Distribute distilled water in quantities of 40 c.c. each in chemically clean, preferably sterile, test tubes. Plug the tubes with gauze-wrapped, nonabsorbent cotton, and sterilize in an autoclave.

Inactivating Fluids for Inactivating Bacteriostatic Agents.—

Fluids A or B are used for removing the various chlorine, iodine, or mercury compounds which are employed as bacteriostatic agents in the tubing fluids used in the packaging of surgical gut. Fluid C is necessary as a preliminary inactivating fluid if the chemical analysis shows that the sutures contain copper salts. If bacteriostatic agents, other than those already designated, are used, appropriate sterile inactivating fluids and appropriate sterile washing agents must be employed so as to remove effectively the bacteriostatic and inactivating agents, which if present would interfere with bacterial growth in the subsequent test for sterility.

Inactivating Fluid A

Sodium thiosulphate	10 gm.
Sodium carbonate	10 gm.
Distilled water, a sufficient quantity to make	1,000 c.c.

Mix, filter if necessary, and distribute in quantities of 40 c.c. each, in chemically clean, dry, sterile test tubes. Sterilize in an autoclave at from 15 to 20 pounds pressure (121° to 125° C.) for from twenty to thirty minutes.

Inactivating Fluid B

Sodium thiosulphate	100 gm.
Distilled water, a sufficient quantity to make	1,000 c.c.

Mix, filter if necessary, and distribute in quantities of 40 c.c. each, in chemically clean, dry, sterile test tubes. Sterilize in an autoclave at from 15 to 20 pounds pressure (121° to 125° C.) for from twenty to thirty minutes.

Inactivating Fluid C

Ammonium chloride	50 gm.
Stronger ammonia water	15 c.c.
Distilled water, a sufficient quantity to make	1,000 c.c.

Dissolve the ammonium chloride in enough distilled water to make 1,000 c.c., filter if necessary and sterilize in an autoclave at from 15 to 20 pounds pressure (121° to 125° C.) for from twenty to thirty minutes. Add 15 c.c. of stronger ammonia water, and distribute the solution aseptically in quantities of 40 c.c. each, in chemically clean, dry, sterile test tubes.

Petrolatum-Paraffin Mixture for an Anaerobic Seal.—

Petrolatum, having a melting point of 48° C.	50 gm.
Paraffin, having a melting point of 57° C.	50 gm.

Liquefy the ingredients by gentle heat and distribute preferably in quantities of 50 or 100 c.c. each in chemically clean, dry test tubes or other containers. Sterilize in a dry heat oven at 170° C. for two hours or by any other suitable method.

Suggested Technic for Conducting Tests for Sterility

Carry out all bacteriologic tests under the most rigid aseptic conditions. Wherever possible, tests should be carried out by two operators working together. The outside wearing apparel of the operators should consist of sterile caps, gowns, and face masks to cover the nose and mouth, and all manipulations should be conducted in a small, dust-proof room supplied with filtered air under positive pressure. The air in the testing room should be sprayed with water and the room itself washed down with a disinfectant each time transfers are to be made, or at least once daily when in use, the room being allowed to remain closed for fifteen minutes thereafter.

Opening Packages and Containers

For Purified Cotton, Gauze, Surgical Dressings, and Related Products.—Flame with care the carton, package, container, or one of the margins if in an envelope, to remove adhering dust particles. Remove material from the package with sterile forceps. Sterilization of the latter is effected in an autoclave or by wrapping the forceps and heating them in a hot-air oven at 170° C. for two hours. Between successive transfers thoroughly flame the forceps. If scissors are needed, sterilize them by the technic directed for forceps.

For Sutures.—If necessary make a file line in the center of the tube or about 10 mm. above any tubing fluid. Then place the tubes in a suitable, active disinfecting solution for 24 hours. Remove the tubes with sterile forceps and place them between sterile towels. As an alternative method of sterilization, flame the tubes, preferably in a wing flame, avoiding the heating of the contents.

Removing and Culturing Contents.—Take portions of the substance in triplicate from the cartons, wrapped packages, envelopes, and similar containers. The material to be tested should be taken from various locations within the roll of purified cotton, gauze, surgical dressing, or related material, preferably from the outer end, center, and core, using sterile instruments and equipment. Transfer these portions of the material, as rapidly as possible, to the necessary number of tubes of medium for aerobic and anaerobic culturing, and also to tubes of honey medium for detecting molds and yeasts. Before use, heat the culture medium for anaerobiosis at 100° C. for fifteen minutes and cool quickly. Seal the inoculated anaerobic medium with a thick (3 cm.) layer of sterile petrolatum-paraffin mixture. Place the sealed tubes immediately in a 37° C. incubator or in a cool water bath to solidify the seal. The time between the removal of the medium from the steam bath and the pouring of the seal should not exceed 15 minutes when examining sutures, and 30 minutes when examining other solids. Cultures of sutures are incubated at 37° C. for 15 days before negative results are recorded. Other products are to be incubated as directed above for 7 days before negative results are recorded. The cultures in honey medium for yeast and fungi are to be incubated for 15 days at from 22° to 25° C. before negative results are recorded.

In the case of sutures, break the tube at the filed line preferably by holding against it a red-hot, curved wire. Transfer the entire suture or loop or strand of gut, with the points of the sterile forceps, to the test tube containing 40 c.c. of sterile distilled water, replace the cotton plug, and incubate the tube at 37° C. for 24 hours. Then transfer the gut to a test tube containing 40 c.c. of sterile inactivating fluid A, using the necessary precautions to prevent contamination, and again incubate at 37° C. for 24 hours.

If a chemical analysis of at least 4 sutures of any lot reveals that the sutures are impregnated with more than 2 per cent of a mercury compound or more than 5 per cent of chlorine or iodine, use sterile inactivating fluid B instead of the above. If the sutures upon chemical analysis have been found to contain copper, transfer the suture, after treatment with 40 c.c. of sterile distilled water to 40 c.c. of sterile inactivating fluid C and again incubate at 37° C. for 24 hours.

It may also be necessary at this point to employ other appropriate sterile inactivating fluids if bacteriostatic agents other than mercury, iodine, or chlorine have been used. If inactivating fluid B or inactivating fluid C, or special inactivating fluids are used, follow by placing the suture in 40 c.c. of sterile inactivating fluid A. That the inactivating fluid used may not interfere with bacterial culturing, an additional step is necessary before the transfer of the suture to the culture medium. This is to wash the suture, after treatment with the inactivator, in another tube of 40 c.c. of sterile distilled water. Finally place the gut in each of the three tubes of medium to test for aerobes, anaerobes, and for molds and yeasts as detailed above.

Confirm all positive cultures showing growth by a microscopic examination of stained smears.

At the end of the period of incubation inoculate at least 5 per cent of all negative aerobic suture cultures showing no growth with 1 c.c. of a 1:100,000 dilution of an eighteen- to twenty-four-hour broth culture of *Esch. coli* and incubate at 37° C. for three days. Inoculate at least 5 per cent of all negative anaerobic suture cultures showing no growth with 1 c.c. of a 1:100,000 dilution of an eighteen- to twenty-four-hour broth culture of *Cl. novyi* and incubate at 37° C. for three days. Inoculate at least 5 per cent of all negative yeast and mold suture cultures showing no growth, with 1 c.c. of a 1:1000 dilution of a seventy-two-hour honey medium culture of *Monilia albicans* and incubate at from 22° to 25° C. for three days. Failure of growth is evidence that bacteriostatic agents which may have been carried over in the transfer are present.

Controls

Tubes of media, distilled water, and inactivating fluid are preferably placed in cans or baskets before sterilization and then covered by paper hoods. Incubate all tubes of culture media immediately after their preparation, for 48 hours at 37° C. and then for 48 hours at room temperature. Discard tubes showing growth. Culture aerobically and also anaerobically 10 c.c. of the distilled water used in these tests and also each of the inactivating fluids, immediately after their preparation. If any of the tubes used in testing show growth the entire stock of material they represent must be discarded.

When testing any material for sterility, the following controls are to be conducted simultaneously:

(a) Inoculate two tubes of each of the culture media to be used with 10 c.c. of the distilled water used and incubate for fifteen days.

(b) Carry out a similar test as in (a) but replace the distilled water with the inactivating fluid used. If more than one inactivating fluid is to be employed, each one must be tested separately.

(c) Seal two tubes of the anaerobic culture medium with a 3 cm. layer of the petrolatum-paraffin mixture and incubate for fifteen days to determine the sterility of the anaerobic seal.

(d) The culture medium to be used for anaerobic culturing should support growth upon inoculating 40 c.c. with 1 c.c. of a 1:100,000 dilution of an eighteen- to twenty-four-hour broth culture of *Cl. novyi.* The culture medium to be used for aerobic culturing should support growth upon inoculating 40 c.c. with 1 c.c. of a 1:100,000 dilution of *Esch. coli.* Incubate inoculated tubes for 72 hours at 37° C. The culture medium used for molds and yeasts should support growth upon inoculating 40 c.c. with 1 c.c. of a 1:1000 dilution of a seventy-two-hour honey medium culture of *Monilia albicans* and incubating at 22° to 25° C. for three days.

Testing for Sterility of Biologic Products

Fluid thioglycollate medium M-48 is used.

Prepare according to the label on the bottle, by suspending 29.5 grams in 1000 c.c. of cold distilled water, heating to boiling to dissolve the medium completely, and distributing into test tubes. Sterilize in the autoclave at 15 to 17 pounds pressure for 18 to 20 minutes.

The final reaction of the medium will be pH 7.1. Store the tubes in the dark at room temperature. Do not use if more than 30 per cent of the uppermost portion of the medium has changed to a pink color. However, one reheating in a boiling water bath is permissible to drive off the absorbed oxygen.

To test for sterility of preparations containing a preservative, the amount of medium used must be sufficient to dilute the inoculum beyond the bacteriostatic limits of the preservative. On the basis of a 1 c.c. inoculum, Difco suggests the amounts of medium to be used for the various types of preservative commonly used in biological preparations, Table 165.

If sterility tests are to be made of certain biologic products which are turbid or otherwise cannot be cultured satisfactorily in fluid thioglycollate medium because of their viscosity, use the formula listed below:

Bacto-N.I.H. Thioglycollate Broth or BBL Sterility Test Broth

Casitone or Trypticase	15 gm.
Yeast extract	5 gm.
Dextrose	1 gm.
Sodium chloride	2.5 gm.
l-Cystine	0.05 gm.
Sodium thioglycollate	0.5 gm.

Suspend 24 gm. in 1000 c.c. cold distilled water. Distribute in Smith fermentation tubes and sterilize in the autoclave for 18 to 20 minutes at 15 to 17 pounds pressure. The final reaction will be pH 7.1±.

In those instances where a solid agar medium is needed for the maintenance of cultures isolated in connection with the sterility testing of a biologic product, or for use as a solid sterility test medium, use BBL or Bacto-N.I.H. agar, dehydrated.

N.I.H. Agar

Casitone or Trypticase	15 gm.
Yeast extract	5 gm.
Dextrose	1 gm.
Sodium chloride	2.5 gm.
l-Cystine	0.05 gm.
Agar	15.0 gm.

Suspend 38.5 gm. of N.I.H. agar in 1000 c.c. cold distilled water. Heat to boiling to dissolve and distribute into tubes or flasks. Sterilize in the autoclave at 15 to 17 pounds pressure for 18 to 20 minutes. Final reaction will be pH 7.1±.

Table 165 gives the minimum quantities of medium for use in sterility tests.

TABLE 165.—MINIMUM QUANTITIES OF MEDIUM FOR USE IN STERILITY TESTS*

PRESERVATIVE USED	TYPE OF BIOLOGIC PRODUCT	AMOUNT OF PRESERVATIVE PRESENT	MINIMUM VOLUME OF CULTURE MEDIUM TO USE PER 1 C.C. OF INOCULUM
Phenol	Sera and vaccines	0.5%	40 c.c.
Cresol, U.S.P.	Serum	0.35%	60 c.c.
Merthiolate	Toxoids Human plasma	1:10,000 1:35,000	10 c.c.
Phenyl mercuric acetate	Human plasma Normal saline	1:50,000 1:100,000	10 c.c.
Phenyl mercuric borate	Human plasma Normal saline	1:50,000 1:100,000	10 c.c.
Phenyl mercuric nitrate	Human plasma Normal saline	1:50,000 1:100,000	10 c.c.
Merthiolate + phenol	Serum	1:10,000 +0.2%	20 c.c.
Chlorbutanol	xxx	0.5%	40 c.c.
Formalin	xxx	0.4%	40 c.c.

*Manual of Dehydrated Culture Media and Reagents, ed. 9, revised 1953, Difco Laboratories, Inc., Detroit, Mich.

THE TESTING OF DISINFECTANTS

The Reddish Method of Testing Solutions for Antiseptic Action

Prepare a twenty-four-hour broth culture of *Staphylococcus aureus*, FDA.

Have on hand No. 2 Whatman filter paper, previously cut into pieces about 0.5 cm. square, and sterilized in the dry heat sterilizer at 170° C. for two hours.

Prepare numerous sterile test tubes with cotton plugs, and sterilize in the dry heat sterilizer. These are to be used for the 5 c.c. of diluted antiseptic used in the test.

Have on hand tubes of nutrient broth M-2, previously tested for sterility. If the material to be tested is oil, and is to be diluted, have sterile mineral oil on hand.

If the material is to be diluted with water, have on hand sterile distilled water.

Prepare 5 c.c. pipettes, graduated 10 c.c. pipettes, and 1 c.c. pipettes, and sterilize.

Technic.—Transfer the sterile paper squares to the twenty-four-hour broth culture of *Staphylococcus aureus*.

Prepare the various dilutions of the disinfectant to be tested, placing 5 c.c. of each dilution into properly marked sterile test tubes.

Determine the time intervals of exposure of the organism to the disinfectant before making the test.

Assume that time intervals are to be thirty seconds, one minute, two minutes, three minutes, four minutes, and five minutes. Each dilution of antiseptic will require 6 transplants. If there are to be three dilutions (undiluted, 1 to 2, and 1 to 10), 18 tubes of nutrient broth are arranged 6 in a row in test tube racks.

18 more tubes of broth are arranged in test tube racks. These are used to shake off the disinfectant. All tubes are properly labeled with the dilution and the time interval.

Proceed with the test:

1. Fish a piece of the paper from the *Staphylococcus aureus* broth culture by means of a flamed platinum wire.
2. Place this immediately in 5 c.c. undiluted disinfectant.
3. At the end of 30 seconds, transfer the paper to a tube of sterile broth.
4. Shake in this broth for 5 minutes to remove the disinfectant.
5. Transfer the paper to a second tube of sterile broth, labeled with the dilution and time interval, and incubate at 37° C. for three days.

Carry out similar tests at different time intervals and different dilutions, incubating all tests at the same time.

At the end of the three-day incubation period, report the results in each tube, as either sterile or growth.

If more than one test organism is to be used, manifestly more material must be prepared.

Food and Drug Administration Methods of Testing Antiseptics and Disinfectants[1]

The Food and Drug Administration method is applicable to the testing of phenol-like disinfectants, such as coal-tar and cresylic acid compounds and other phenolic preparations, and gives results quite comparable to those obtained with the Hygienic Laboratory method which is limited only to phenol-like compounds. It must be distinctly understood that this method is not well adapted for the testing of chlorine compounds or for oxidizing agents in general since the presence of organic matter adversely affects the germicidal effectiveness of such preparations under practical conditions. Therefore, a phenol coefficient figure for chlorine compounds would be misleading to the consumer since such a figure would not indicate the actual germicidal efficiency of such disinfectants under practical conditions in the presence of organic matter.

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[1] Ruehle, G. L. A., and Brewer, C. M.: Circular No. 198, December, 1931, United States Department of Agriculture, Washington, D. C.

Apparatus and Technic

''**Test Organism and Culture Medium.**—The test organism is a 22- to 26-hour culture of *Salmonella typhosa* (Hopkins strain) incubated and grown in nutrient broth at 37° C. The broth contains the following ingredients: 5 gm. of Liebig's beef extract, 5 gm. of chemically pure sodium chloride, and 10 gm. of Armour's peptone (for disinfectant testing) in 1,000 c.c. of distilled water. The mixture is boiled for 20 minutes, made up to original weight (or volume) with distilled water, and adjusted with NaOH to pH 6.8 using the colorimetric method. It is then filtered through paper, tubed (10 c.c. to each tube), and the tubes are plugged with cotton and sterilized at 15 pounds pressure for 40 minutes. The test culture is transferred daily in this medium for not more than one month. At the end of each month, a fresh transfer is made from the stock culture. The stock culture is carried on agar slants of the same composition as the broth medium plus 1½ per cent Bacto-Agar (Difco), adjusted to pH 7.2 to 7.4. This medium is also filtered, tubed, plugged with cotton, sterilized, and slanted. The stock culture is transferred once a month, and the test organism is taken from the month-old stock culture. When the test organism has not been transferred daily, it is advisable to make four or five consecutive daily transfers in broth before using it for testing purposes, to be reasonably sure of its conforming to the phenol resistance requirements. When only one transfer has been skipped the following transfer from the 48-hour culture is usually satisfactory for use after 24 hours. Transfers are made with the platinum loop used in the test. Only cultures giving readings within the following limits are considered satisfactory:

Phenol:	5 minutes	10 minutes	15 minutes
1- 90	+	+	0
1-100	+	+	+
or			
1- 90	0	0	0
1-100	+	+	0

''The following reading is that most usually obtained and is the most convenient:

Phenol:	5 minutes	10 minutes	15 minutes
1- 90	+	0	0
1-100	+	+	+

''**Phenol.**—The phenol used must meet the requirements of the United States Pharmacopoeia, and in addition the congealing point must not be below 40° C. A 5 per cent solution may be used as a stock solution if kept in a relatively cool place in well-stoppered amber-colored bottles protected from the light. This 5 per cent solution should be standardized with decinormal bromine or with sodium bromide and bromate solution.

''**Apparatus.**—Besides a number of accurately graduated pipettes, 100 c.c. glass-stoppered graduates or volumetric flasks are almost essential for the making of correct dilutions. All pipettes and graduates should be standardized. The test tubes for containing the dilutions should be large enough to permit transfers being made without touching the sides with the transfer needle. Lipped Pyrex (to withstand constant flaming) test tubes 25 by 150 mm. serve very well as these seeding or medication tubes. A water bath for holding the dilutions at the desired temperature must be provided. To maintain the temperature practically constant during the period of the test, the bath should be made so as to contain a relatively large volume per surface area, and should be insulated. The lid is made with well-spaced holes admitting the 25-mm. tube, but not the lip. The most convenient form of subculture tubes (tubes containing medium for incubating the tested organisms, as well as for growing the test culture) are ordinary nonlipped bacteriological test tubes 20 by 150 mm. The racks for holding the subculture tubes may be any convenient style. Blocks of wood with a series of holes bored in them are quite satisfactory. Dimensions depend somewhat on the size of the incubator, but the holes should be well spaced to insure quick selection and easy manipulation during the test. It is an added convenience to have the holes large enough to admit the medication tubes while dilutions are being made. The transfers are made with a 4-mm.

(inside diameter) single loop of number 23 B. & S. gauge platinum wire, 1½ to 3 inches long, set in a suitable holder such as an aluminum or glass rod approximately 0.5 cm. in diameter.

"**Procedure.**—One per cent stock dilutions of the substance to be tested (or any other convenient dilution of the disinfectant, depending on the strength) are made up, usually in the glass-stoppered cylinders or volumetric flasks from which the individual dilutions are then prepared. For rapid routine work, the final dilutions may be made directly in the medication tubes. In this case all excess over 5 c.c. must be removed. For more precise work and when high dilutions are required or volatile substances are dealt with, it is preferable to make up all of the dilutions in volumetric flasks and then transfer 5 c.c. of the final dilution to the medication tubes. These tubes containing 5 c.c. of each dilution (including the phenol control) are placed in the water bath at 20° C. for five minutes until the temperature of the bath is reached. Even slight variations in temperature may affect the results. The dilutions should cover the range of the killing limits of the disinfectant within 5- and 15-minute periods and should at the same time be spaced sufficiently close together to insure the desired accuracy. Five-tenths of a cubic centimeter of the test culture is then added to each of the dilutions at a time interval corresponding to the interval at which the transfers are to be made. Thus by the time 10 tubes have been seeded at 30-second intervals, four and one-half minutes will have elapsed and a 30-second interval intervenes before the transference to the subcultures is commenced. The culture is added from a graduated pipette holding sufficient culture to seed all the tubes in any one set. The pipette may be loosely plugged with cotton at the mouth end before sterilizing, as a precautionary measure. Unfiltered culture is used, but it should be thoroughly shaken 15 minutes before use and allowed to settle. The temperature of the culture should be practically that of the water bath before being added.

"In inoculating the medication tubes they should be held in a slanting position, after removal from the bath, and the culture run in without the tip of the pipette touching the disinfectant. The tip may be allowed to rest against the side of the tube just above the surface of the liquid. The tubes are agitated gently but thoroughly after the addition of the culture to insure even distribution of the bacteria. Five minutes from the time of seeding the first medication tube, transfer 1 loopful of the mixture of culture and diluted disinfectant from the medication tube to the corresponding subculture tube. To facilitate transfer of uniform drops of the medication mixture, the loop is bent to form a slight angle with the stem and the medication tube is held at an angle of 60°. In other words, as the loop is withdrawn, its plane should be parallel with the surface of the liquid. At the end of 30 seconds, a loopful is transferred from the second medication tube to the second subculture tube and the process is continued for each successive dilution. Five minutes from the time of making the first transfer, a second set of transfers is begun for the 10-minute period and finally repeated for the 15-minute period. Before each transfer the loop is heated to red heat in the Bunsen flame and the mouth of every tube is flamed. Sterilization of the loop is effected immediately after making the previous transfer (before replugging the tubes) to allow time for sufficient cooling. Time does not permit flaming the tubes after making the transfer. For this reason, care in transferring and seeding is necessary. Due caution is observed to prevent either the seeding pipette or the transfer needle from touching the sides or mouth of the medication tube; neither should cotton threads be found adhering to the sides or mouth of these. After completion of the transferring, the subculture tubes are incubated at 37° C. for 48 hours and results are read. Macroscopic examination usually suffices for this, but occasionally agglutination with antityphoid serum will aid in reading doubtful results. A 3-day incubation period or agar streak or microscopic examination may be resorted to in determining feeble growth, especially when organisms other than *Salmonella typhosa* are used.

"There are certain types of germicidal agents, such as many of the mercury compounds, which give very high results by phenol coefficient tests.[2] Due to the high inhibitory value of such substances in preventing growth in the subcultures these figures are frequently misleading. For germicides used in the disinfection of such objects as surgical instruments, this is of particular importance and must be taken into account. Failure to appreciate this characteristic of certain compounds is much more likely to lead to error when *Staphylococcus aureus* is used rather than *Salmonella typhosa* as the test organism. That false values may not be obtained for products of this type, or for any other disinfectant giving suspiciously high results, the subcultures should contain very large amounts of medium (not less than 200 c.c.) or they should be retransferred by carrying at least 4 loopfuls from the first subculture to a second tube of broth, as recommended by Shippen.*

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[2] Am. J. Pub. Health 18: 1231, 1928.

''Other groups of disinfectants in common use, for which the phenol coefficient method of testing is not well adapted, are those compounds containing chlorine as the active agent as well as oxidizing agents in general. These are affected so materially by the presence of organic matter that a phenol coefficient statement may grossly misrepresent their value under practical conditions of use and is very apt to be misleading to the consumer when placed on the label.''

Calculation of the Phenol Coefficient

''The results of the test are expressed in terms of the *phenol coefficient.* This represents the germicidal value of the diluted disinfectant as compared with the diluted phenol control. It is a figure obtained by dividing the numerical value of the greatest dilution (the denominator of the fraction expressing the dilution) of the disinfectant capable of killing *Salmonella typhosa* in 10 minutes but not in 5 minutes, by the greatest dilution of phenol showing the same results; that is, by the phenol control. Thus, if the results were as follows:

	5 minutes	10 minutes	15 minutes
Disinfectant (X):			
1-300	0	0	0
1-325	+	0	0
1-350	+	0	0
1-375	+	+	0
1-400	+	+	+
Phenol:			
1- 90	+	0	0
1-100	+	+	+

the phenol coefficient would be $\frac{350}{90} = 3.89$.

''If none of the dilutions shows growth in 5 minutes and killing in 10 minutes, the hypothetical dilution may be estimated in certain cases. This may be done only when any three consecutive dilutions show the following results:

''The first, no growth in 5 minutes; the second, growth in 10 minutes but not in 15 minutes; and the third, growth in 15 minutes; for example:

''If the results were as follows:

	5 minutes	10 minutes	15 minutes
Disinfectant (X):			
1-300	0	0	0
1-350	+	+	0
1-400	+	+	+
Phenol:			
1- 90	0	0	0
1-100	+	+	0

the estimated phenol coefficient would be $\frac{325}{95} = 3.42$.

''To avoid giving an impression of fictitious accuracy, the phenol coefficient is calculated to the nearest 0.1 unless the coefficient is less than 1.0. Thus, in the examples cited above, the phenol coefficients would be reported as 3.9 and 3.4 instead of 3.89 and 3.42.

''In the preceding description, *S. typhosa* has been mentioned as the test organism. Wherever any expression of phenol coefficient occurs in literature, on labels, etc., it is assumed to mean the *S. typhosa* phenol coefficient, unless otherwise stated. It is, however, the distinct intention of this department* not to limit the test to the use of one organism. In fact, the test has been found adaptable to the use of a wide variety of bacterial species in the determination of phenol coefficients. In cases where some of the more strictly parasitic bacteria are used, modifications in media are necessitated, and, of course, a change in the phenol dilutions. The writers are not in a position at this time to prescribe the limits of resistance for many of the organisms that might be used. Therefore discussion of the exact technic is here omitted, with the exception of that for *Staphylococcus aureus.* Suggestions for the use of certain representative types may, however, be found in a paper by Reddish.† When any test organism other than *S. typhosa* is used it should be distinctly designated when stating the phenol coefficient.

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*Loc. cit., p. 1704

''*Staph. aureus* has been found to be an extremely useful organism for testing disinfectants and antiseptics and has been used for this purpose for a number of years. When substituted in the above test the technic remains exactly the same. The phenol dilutions, however, must be changed. The resistance of any strain of *Staph. aureus* used in this test must come within the following limits: At 20° C. it must survive a 1:60 dilution of phenol for 5 minutes and a 1:70 dilution for 15 minutes. The following is the minimal resistance that would be acceptable:

	5 minutes	10 minutes	15 minutes
Phenol:			
1-60	+	0	0
1-70	+	+	+

''In the bacteriological examination of disinfectants, the *S. typhosa* and the *Staph. aureus* phenol coefficients give, in general, sufficient information to render tests with other organisms unnecessary, except in special instances. The commonly accepted criterion that disinfectants for general use be employed at a dilution equivalent to the germicidal efficiency of 5 per cent phenol against *S. typhosa* (that is, 20 times the *S. typhosa* phenol coefficient) allows a reasonable margin of safety for the destruction of infective agents likely to be the object of general disinfection about premises with the possible exception of *Mycobacterium tuberculosis. Staph. aureus,* due to its ubiquity, resistance and ever-ready tendency to cause infection, should always be employed in testing those substances recommended for personal use or as applications for wounds. If the disinfectant is recommended for use externally the temperature of test should be 20° C., but where such substances are recommended for use in the body cavities, such as for mouth washes, gargles, douches, etc., this test should be conducted at 37°. In such case the test should be designated 'The F.D.A. method (special) *Staph. aureus,* 37° C.' At body temperature the *Staph. aureus* should show the following resistance to phenol:

	5 minutes	10 minutes	15 minutes
1-80	+	0	0
1-90	+	+	+

Or

	5 minutes	10 minutes	15 minutes
1-80	+	0	0
1-90	+	+	0

''

Reddish emphasizes that antiseptics are chemical agents used to kill or inhibit infectious bacteria on the human and animal body, and that the phenol coefficient test is not suitable for testing antiseptics and should never be used for this purpose. A figure showing germicidal activity against *S. typhosa* does not indicate the effectiveness of antiseptics against pus-producing and other infectious bacteria which are present in and on the body. It is obvious that antiseptics should be tested against the kind of bacteria which they are expected to kill under practical conditions. Reddish in 1925[1] published a method for testing soluble liquid antiseptics against *Staphylococcus aureus* of known normal resistance to phenol. In this test 0.5 c.c. of a 24-hour F.D.A. broth culture of *Staphylococcus aureus* is added to 5.0 c.c. of antiseptic at 37° C., and transfers are made from this mixture to 10 c.c. of sterile broth after 5 minutes (officially designated as the F.D.A. method *Staphylococcus aureus* 37° C., 18). If all of the microorganisms (approximately 350,000,000) in the 0.5 c.c. of culture are killed by 5.0 c.c. of antiseptic in 5 minutes, the product is an effective antiseptic. The culture of *Staphylococcus aureus* employed in this test must not be killed by 1:80 phenol in 5 minutes nor by 1:90 phenol in 10 minutes at 37° C. under the conditions of the test as just outlined. (No comparison of any kind is made to the results obtained with phenol, the phenol test being only a control on the resistance of the culture.) Such antiseptics are approximately equal in germ-killing power to the 1:40 solution of carbolic acid used in antiseptic surgery. Antiseptics which pass this test are effective germicides when used in practice, as has been proved in the case of antiseptics for oral use.[2]

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*United States Department of Agriculture, Washington, D. C.

†Am. J. Pub. Health 17: 320, 1927.

The classes of preparations to which this test is applicable are the various germicidal solutions ordinarily used for first aid, for oral use, and for general applications about the body for the prevention of infections. Tincture of iodine, liquor antisepticus, aqueous solutions of iodine, solutions of phenol, mercury compounds, essential oils, halogen compounds, etc., should be tested by this method. Solutions of antiseptic dyes, however, should be tested by the agar cup plate method.[3] The test is not especially applicable for the testing of antiseptics for skin disinfections, as has been shown by Tinker and Sutton,[4] Reddish and Drake,[5] and Simmons.[6] The F.D.A. method *Staphylococcus aureus* 37° C. is applicable to those soluble liquid antiseptics which are employed for short periods of contact, and for this purpose it has proved both practical and satisfactory.

Interference of Phospholipids With Phenol Coefficient Results

There are certain pitfalls in all the methods for determination of phenol coefficients of disinfectants, as pointed out by Brewer.[7] He definitely proved that the peptone component of the medium for culturing phenol coefficient test organisms may seriously affect the phenol coefficient figure obtained. This effect on the test culture is not detectable from the results of the phenol coefficient. This work is based upon certain preceding observations. For instance, Petroff and Schain[8] showed that the bactericidal properties of germicides are enhanced by the addition of detergents. For this reason, many manufacturers have been adding detergents or wetting agents to disinfectants, with a consequent increase in the phenol coefficient figure. The result of the killing power against *S. typhosa* depends upon the type of peptone used in the test. The phospholipid content in the peptone is the pivotal point in this matter. Some peptones, for instance, contain a certain amount of phospholipid, others little or none. Their presence and amount determine the results of the test.

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[1] Am. J. Pub. Health **15**: 534, 1925.
[2] Reddish, G. F.: J. Am. Pharm. Assn. **25**: 1117, 1936.
[3] Reddish, G. F.: J. Am. Pharm. Assn. **17**: 237, 1929.
[4] J. A. M. A. **87**: 1347, 1926.
[5] J. A. M. A. **91**: 712, 1928.
[6] J. A. M. A. **91**: 704, 1928.
[7] Am. J. Pub. Health **33**: 261, 1943.
[8] Quart. Bull. Sea View Hosp. **4**: 372, 1940.

What is the answer? What can we do to make accurate and reliable and trustworthy phenol coefficient test results in testing disinfectants containing a detergent? This phospholipid factor, which is the determining point, according to Brewer's work, is present in the standard or recommended peptone (the F.D.A. method prescribes Armour peptone) in favorable amounts. Brewer states "the problem is not easy of solution." He concluded that when tested against *S. typhosa* the germicidal activity of disinfectants containing synthetic detergents is greatly altered by the phospholipid content of the culture medium.

DISEASES DUE TO RICKETTSIAE

A number of diseases, most of which are typhus-like, have been shown to be due to the type of organisms which have been given the name of Rickettsiae. These organisms have been shown in the tissues of many insects and also in the tissues of animals or human beings during certain specific diseases of which they are suspected of being the causative factors. Rickettsiae are small, nonmotile, coccoid-like bodies, about 0.5 micron in diameter, occasionally seen in pairs of bacillary and filamentous forms. They may be demonstrated by using diluted Giemsa stain, 1 c.c. to 50 c.c. distilled water, after fixation for one-half hour in alcohol. The organisms take on a reddish purple coloration with this stain.

Pinkerton* states that present evidence indicates that the rickettsiae, although they are as strictly dependent upon intracellular conditions as the smaller viruses, have a somewhat more complex enzyme system than the latter, and are therefore able to maintain a certain amount of independent metabolic activity within their host cells. He believes that we may consider rickettsiae both from the point of view of size and from the point of view of independent metabolic activity as occupying a position intermediate between certain cytotropic bacteria and the viruses.

Using the criteria suggested by Cowdry† and Wolbach,‡ Pinkerton* defines rickettsiae as small, often pleomorphic, gram-negative, bacterium-like organisms, living and multiplying in arthropod tissues, behaving as obligate intracellular parasites, and staining lightly with aniline dyes. With but a few exceptions, criteria adequate for classification on the basis of biologic properties are available only for those members of the group which are pathogenic for mammals.

The most important diseases which have been shown to be associated with rickettsiae are typhus on the European continent and Rocky Mountain spotted fever on this continent. Ricketts and Wilder[1] first pointed out the etiologic agent of typhus in lice fed upon typhus patients. This was confirmed by da Rocha-Lima,[2] who called the organism *Rickettsia prowazeki*. Wolbach, Todd, and Palfrey[3] experimentally confirmed this etiology by showing

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*Bact. Rev. **1**: 37, 1942.

†J. Exper. Med. **37**: 431, 1923.

‡J. A. M. A. **84**: 723, 1925.

[1] J. A. M. A. **54**: 1373, 1910.

[2] Berl. klin. Wchnschr. **53**: 567, 1916.

[3] Harvard University Press, Cambridge, Mass., 1922.

that lice, originally free from rickettsiae, acquired numerous rickettsiae which distended the intestinal lining cells, when fed on typhus patients. They showed intracellular rickettsiae in the lesions of man and experimental animals, and, further, when the intestines of lice, containing intracellular rickettsiae, were emulsified and injected into guinea pigs, typhus resulted, whereas injection of the intestines of rickettsia-free stock lice produced no illness. The hosts for *Rickettsia prowazeki* are *Pediculus humanus, Xenopsylla cheopis,* and *Ceratophyllus fasciatus.* Rocky Mountain spotted fever, found so frequently in this country, is due to rickettsiae called *Dermacentroxenus rickettsii.* The hosts for this rickettsia are *Dermacentor andersoni, Dermacentor variabilis, Haemaphysalis leporis-palustris, Amblyomma americanum,* and *Rhipicephalus sanguineus.*

Rickettsia Prowazeki (Typhus Rickettsiae).—In paraffin sections, *R. prowazeki* are always intracellular. In smears, extracellular organisms are often seen, as a result of the rupture of cells in the process of making the preparation. They appear frequently as minute diplobacilli, each member of the diploid form averaging about $0.6 \times 0.3\ \mu$. Short chains are not uncommon; sometimes, long chains are seen in tissue culture.

They stain poorly with most aniline dyes. They are gram-negative. They can be stained by the Giemsa method, by Macchiavello's (S-46), or by the Castañeda's stain (S-49).[4] With the Giemsa stain, the rickettsiae are stained blue or purple, with the cytoplasm a pale blue. With the last method, the rickettsiae are stained red and stand out sharply against a blue cytoplasmic background. Stains for Rickettsiae are discussed on pages 1413 and 1711.

Typhus rickettsiae multiply exclusively in the cytoplasm of cells in tissue cultures, and are never seen in nuclei. This behavior is in striking contrast to that of spotted fever rickettsiae as pointed out by Pinkerton and Hass.[5] During mitotic division of their host cells, the organisms are markedly reduced in number and tend to collect at the poles of each cell.[6] When fat droplets appear within cells in tissue culture, the rickettsiae are never seen within these droplets, but crowd the cytoplasm around the droplets.

R. prowazeki are readily killed by heat, drying, or chemical agents. At −20° C., a brain or spleen from a typhus guinea pig, if sealed in a test tube, retains its virulence with a gradual loss of titer for periods ranging up to eight months.[7] Nigg[8] showed that typhus rickettsiae in sealed Maitland cultures at 37° C. are virulent for periods ranging up to several months.

According to Pinkerton[9] the most satisfactory animal for the study of typhus is the guinea pig, in which both murine and human typhus causes a febrile but nonfatal illness. Two distinct types of *R. prowazeki* are recognized. Pinkerton believes that the differences between these two types are of subspecific magnitude and the two types should be given the variety names *R. prowazeki* var. *prowazeki* and *R. prowazeki* var. *mooseri.* The former is the etiological agent of louse-borne human typhus, of the type which has occurred in great epidemics on the European continent, and has been called European or epidemic typhus. *R. prowazeki* var. *mooseri* is the name applied to the etiological agent of a somewhat milder, usually endemic, form of the disease occurring in various parts of the world, and apparently enzootic in rats. This form of the disease is carried from rat to rat by the rat louse (and by the rat flea) and from rat to man by the rat flea. It is called murine typhus.

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[4] Zinsser, H.: Harvard Symposium, Harvard University Press, p. 896, 1940.

[5] J. Exper. Med. **56**: 151, 1932.

[6] J. Exper. Med. **54**: 307, 1931.

[7] Pinkerton, H.: Unpublished observations.

[8] J. Exper. Med. **61**: 17, 1935.

[9] Bact. Rev. **6**: 37, 1942.

Pinkerton states that the difference between these two types of *R. prowazeki* may be summarized as follows:

1. *R. prowazeki* var. *mooseri* after intraperitoneal injection in rats causes a febrile illness, while *R. prowazeki* var. *prowazeki* produces only an inapparent infection. Male rats infected with *R. prowazeki* var. *mooseri* suffer a mild inflammation of the tunica vaginalis with many serosal cells laden with rickettsiae while *R. prowazeki* var. *prowazeki* causes no such reaction.
2. *R. prowazeki* var. *mooseri,* when introduced per anum into *Pediculus humanus,* kills this arthropod in a few days, while *R. prowazeki* var. *prowazeki* requires about three weeks.
3. If injected intraperitoneally in male guinea pigs, *R. prowazeki* var. *mooseri* almost constantly induces an acute inflammation of the scrotal sac with numerous visible organisms, while *R. prowazeki* var. *prowazeki* either fails to cause such a reaction at all or produces it irregularly and in a milder, more transient form.
4. Zinsser's studies with Castañeda brought out certain minor immunologic differences between the two varieties.
5. The most important of all distinguishing features from a practical point of view is the fact that *R. prowazeki* var. *prowazeki* multiplies much less luxuriantly, not only in living experimental animals with resistance reduced by various methods, but also in the various media containing living or surviving cells.

Agglutination Reactions.—There is no true biologic relationship between rickettsiae and *Proteus vulgaris.* Castañeda[10] believes that the production of agglutination of *Proteus vulgaris* by rickettsial sera depends on the possession of a common carbohydrate antigen by the two organisms. In typhus, agglutination is usually found in relatively high titer with the OX19 strain of *P. vulgaris,* and in low titer with the OX2 strain. This enables one to differentiate typhus from tsutsugamushi disease, since in the latter the agglutination titer is characteristically high with OXK. In spotted fever, agglutination occurs with OX19 *or* OX2. Complement fixation is the preferred method.

Diagnostic Methods.—

The complement fixation test gives excellent results. Intraperitoneal injection of 5 c.c. of the patient's blood into each of two guinea pigs during the first few days of illness will result in a febrile disease with involvement of the scrotum or scrotal sac. This is a diagnostic test of typhus or spotted fever. After sacrificing the pigs and making smears from the scrotal sac, a presumptive diagnosis can usually be made, since in typhus we find cells packed with rickettsiae, while in spotted fever the organisms are fewer and larger, and tend to have the characteristic lanceolate form. The nature of the infection can further be proved by carrying out cross-immunity tests with known strains of typhus and spotted fever rickettsiae.

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[10] J. Exper. Med. 62: 289, 1935.

Diagnosis can be made also by cross-protection tests with the patient's serum.

Dermacentroxenus Rickettsi (Spotted Fever Group).—*Dermacentroxenus rickettsi* is the etiologic cause of a number of diseases of the spotted fever group. Wolbach[11] assigned it to a different genus although it resembles in many respects *Rickettsia prowazeki.* Originally it was thought that the disease was localized to the general region of the Rocky Mountains and so it was called Rocky Mountain spotted fever. It has since been found that diseases similar to this are found in all parts of the world. Ricketts[12] and King[13] independently announced in 1906 the transmission of spotted fever to guinea pigs by ticks. Ricketts noted the occurrence of large numbers of organisms in the ova of ticks, and showed that these organisms were agglutinated by immune serum from human beings and guinea pigs. He described the organism in blood films from guinea pigs and man. Wolbach[11] named the etiological agent *Dermacentroxenus rickettsi* and differentiated between this organism and nonpathogenic organisms in ticks. He was the first to demonstrate the intranuclear multiplication of the organism in tick tissues. The etiological relationship was further confirmed by cultivation in tissue culture. Spencer and Parker[14] demonstrated that the "virus" of this disease could exist in ticks in an inactive form but it seems probable, according to Pinkerton, that rickettsiae in noninfective ticks are the nonpathogenic rickettsiae which are often found in ticks and which resemble *D. rickettsi* morphologically. These nonpathogenic rickettsiae are never found within cell nuclei, however, and are believed to be distinct from *D. rickettsi.*

D. rickettsi are about the same size as *R. prowazeki.* Filtration through Berkefeld filters makes the material containing them noninfectious. The **staining characteristics** of *D. rickettsi* are similar to those of *R. prowazeki.* The Giemsa method gives excellent results. A modification of the Macchiavello method was found in Pinkerton's laboratory to be unsatisfactory for staining spotted fever rickettsiae. There are two modifications of the Macchiavello method that stain *D. rickettsi* very sharply and deeply in sections of the guinea pig testicle. By the following modification, the organisms stain deep red and are clearly distinguishable, even though the surrounding tissue is also partly red.

Staining Methods

Staining methods for Rickettsiae are described in full on page 1413.

A good additional routine is:

Fix tissues in Regaud's fluid S-45, cut thin paraffin sections and run through xylol and graded alcohols to distilled water in the usual way.

Stain in 1% aqueous methylene blue overnight.

Decolorize in 95% alcohol.

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[11] J. Med. Research 41: 1, 1919.

[12] J. A. M. A. 47: 358, 1906.

[13] Pub. Health Rep. 21: 863, 1906.

[14] U. S. P. H. S., Hyg. Lab. Bull. No. 154, 1930.

Counterstain in 0.25% aqueous basic fuchsin for 30 minutes.
Decolorize rapidly (about 3 seconds) in 0.5% citric acid.
Differentiate rapidly in absolute alcohol.
Clear in xylol and mount in gum dammar.

The second modification stains the rickettsiae deep blue against a background which is partly red and partly blue:

Procedures are the same as above, through decolorizing in 0.5 per cent citric acid.
Wash lightly in distilled water.
Counterstain again in 1% aqueous methylene blue for 5 seconds.
Differentiate in 95% alcohol.
Dehydrate in absolute alcohol and clear in xylol. Mount in gum dammar.

The essential lesion of spotted fever is a specific endangiitis. Focal brain lesions have been found in spotted fever guinea pigs which do not die in the earlier stages of the disease. Monkeys, rabbits, and guinea pigs are susceptible to spotted fever; the pathologic picture in these animals is the same as in man. Dogs are susceptible to infection with fièvre boutonneuse.

The diseases recognized as belonging to the spotted fever group are as follows: 1. Rocky Mountain spotted fever; 2. Eastern spotted fever; 3. Fièvre boutonneuse; 4. São Paulo typhus; and 5. South African tick-bite fever.

Either Proteus OX19 or Proteus OX2 is agglutinated.

The results of complement fixation tests are excellent, but cross reactions with rickettsialpox are the rule.

Rickettsia Tsutsugamushi.*—This is a rickettsial disease due to the *Rickettsia tsutsugamushi.* It was transmitted by Ishiwara and Ogath[15] to the rabbit by intratesticular inoculation. *R. tsutsugamushi* is similar in its morphology to *R. prowazeki* and *D. rickettsi.* It is a short diplobacillus with bipolar staining. It is shorter and plumper than typhus and spotted fever rickettsiae. Ogata and Unno[16] showed short chains. It stains like the other rickettsiae but shows bipolar staining more prominently.

In cultivation work, it grows more freely in the cytoplasm of the interstitial cells of the rabbit testis and the endothelial cells overlying Descemet's membrane than the other organisms in this class. The lesion is essentially that of an endangiitis, with ascites in infected guinea pigs; rickettsiae are found in cells of the ascitic fluid. Rodents are the reservoir.

The Weil-Felix reaction is characterized by agglutination of *Proteus vulgaris* OXK in high serum titer. Complement fixation is not used because no antigen is available.

Rickettsia Wolhynica (Trench Fever).—The body louse was found to transmit trench fever in man during the world war of 1914-1918. It can be transmitted to human volunteers by rubbing the feces of infected lice into the scarified skin and also by allowing infected lice to feed on the volunteers. The evidence of the etiological relationship of this organism to trench fever has not been completely described. There has been an utter failure in inducing infection in experimental animals.

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*Scrub typhus; Japanese river fever.

[15] Tokyo Ijishinshi, No. 2581: 1555, 1928.

[16] Mitt. med. Ges. Chiba 7: 1929.

R. wolhynica is more deeply stained in film preparations than the more characteristic rickettsiae. The organisms are also somewhat plumper, more definitely oval, and less pleomorphic than typhus rickettsiae.

Coxiella Burnetii (Diaporica). (Q Fever).—Burnet and Freeman[17] described a rickettsia-like organism in smears from the spleens of infected mice, in studying an epidemic among individuals working in abattoirs or on dairy farms in Australia. It was called *Rickettsia burnetii* by Derrick.[18] It was found to be pathogenic for guinea pigs. This organism was also called *Rickettsia diaporica.* The relationship of *C. burnetii* and *C. diaporica* to Q fever in Australia and in the United States has been established by guinea pig inoculation and serologic tests.

This organism is filtrable, hence the name "diaporica." Morphologically the rickettsiae of Q fever are similar to those of spotted fever and typhus, but appear somewhat larger because they stain more deeply. They grow in various media containing living cells commonly used for the growth of rickettsiae and filtrable viruses.

The essential pathologic findings in man are pulmonary edema and congestion. It is an atypical pneumonia. Similar pathologic changes were seen in the lungs of experimentally infected monkeys. Guinea pigs have been successfully infected by the inoculation of human blood or urine.

Proteus OX19, OX2, and OXK are not agglutinated by the serum of the patient, but complement fixation is of great value.

North Queensland tick fever gives a positive agglutination test with Proteus OX2. Complement fixing antibodies appear and are specific. The animal reservoir is unknown.

Rickettsialpox is a mite-borne tenement disease, carried by mites to man from mouse. It is caused by *Rickettsia akari.* Protei are not agglutinated by the serum of the patient, but complement fixing antibodies cross-reacting with Rocky Mountain spotted fever are present.

Weil-Felix Reaction

There is an agglutination reaction, known as the Weil-Felix reaction, performed on the serum of typhus patients, which is considered diagnostic of typhus. This reaction takes place between the patient's serum agglutinins and certain varieties of Proteus organisms, which though isolated from typhus cases are presumably not the cause of the disease. These organisms include the *Proteus X* strains of Weil and Felix. These organisms are nonmotile, produce indol, and acid and gas from glucose, maltose, and saccharose, but not from lactose or mannitol. They digest gelatin and blood serum somewhat more slowly than typical cultures of *Proteus vulgaris.* The reaction appears during the first week of the disease but becomes quite marked in the second week and during convalescence. In nonvaccinated individuals, a titer of 1:160 or higher is significant.

Technic.—

The *Proteus X* cultures are grown on agar slants, pH 7.4, then thinly inoculated on the dry surface of agar in Petri plates, and incubated for 24 hours at 37° C. From these plates smooth, nonspreading "O" type colonies are transferred to tubes of broth and slanted agar. If at the end of 24 hours' incubation the cultures are nonmotile, they are used in preparation of the antigen O. The living organisms are suspended in physiologic salt solution until a standard degree of turbidity is produced corresponding to tube No. 3 of the McFarland nephelometer.

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[17] M. J. Australia 2: 299, 1937.

[18] M. J. Australia 1: 14, 1939.

A macroscopic agglutination test is now carried out.* Take 2 to 5 c.c. of the patient's blood by venous puncture and separate the serum from the clot.

Arrange 6 small test tubes in a rack.

In the first tube place 2.3 c.c. of 0.85% saline and 1 c.c. in all the remaining tubes.

Next, to tube No. 1 add 0.2 c.c. of serum.

Mix, transfer 1 c.c. to tube No. 2 and discard 0.5 c.c.

Mix the contents of tube No. 2 and transfer 1 c.c. to tube No. 3.

Continue this until the last tube, from which 1 c.c. is discarded.

TABLE 166

NAME OF DISEASE	LOCALITY	VECTOR	AGGLUTINATION WITH PROTEUS X TYPES			COMPLEMENT FIXATION TEST
			OX19	OX2	OXK	
Epidemic typhus	Old and New Worlds	Lice	xxx	x or –	–	Used
Endemic typhus	Old and New Worlds	Rat fleas and lice	xxx	x or –	–	Used
Brill's disease (recrudescence)	Europe, U.S.A.		x	–	–	Used
Tsutsugamushi (scrub typhus)	Far East	Mites	–	–	xxx	
Rocky Mountain spotted fever	U.S.A.	Ticks	xxx or –	– or xxx	–	Used; cross-reaction with rickettsialpox
Fièvre boutonneuse	Mediterranean	Ticks	x	x	–	?
South African tick-bite fever	South Africa	Ticks	x	x	x	?
North Queensland tick typhus	Australia	Ticks	–	xxx	–	Used
Q fever	Old and New Worlds	Varies	0	0	0	Used
Rickettsialpox	U.S.A.	Mites	0	0	0	Used; cross-reaction with Rocky Mountain spotted fever

xxx—Main agglutination. x—Group agglutination. ?—Not tested adequately.

To all tubes add 1 c.c. of antigen and shake well. The final dilutions of serum will now be 1:25, 1:50, 1:100, 1:400, and 1:800.

Incubate the tubes at 50° to 55° C. for 2 hours and place in the refrigerator overnight. Read only 2+ or stronger agglutinations.

Controls should be carried out using a known positive human or rabbit immune serum; also tests employing a known normal human serum.

The agglutination titer of sera from typhus cases may vary from 1 to 50 to 1 to 50,000 while the titer of normal sera is rarely higher than 1 to 25 or 1 to 50. Agglutination with low titers may be of diagnostic significance if found early in the disease, especially if they follow a negative reaction.

Rapid slide tests are described on page 1529.

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*See p. 1529 for the febrile antigens—rapid macroscopic agglutination tests.

Gintcheff Slide Agglutination Test.—

The Gintcheff method uses dried blood collected on a slide. For Gintcheff's method, prepare a suspension of Proteus OX19 in 1.5% sodium citrate. Filter the suspension through a few layers of gauze and heat it for 30 minutes at 80° C. on a water bath.

To each 100 c.c. of the suspension, add 0.2 c.c. of Formalin and 0.5 c.c. of a saturated solution of malachite green *or* of a saturated alcoholic solution of fuchsin. Filter the mixture again.

Add one drop of the suspension to a drop of dried blood on a slide and mix. Formation of small flakes is equivalent to a 1:50 positive reaction in the tube test, while large flakes indicate a reaction corresponding to a positive reaction of a 1:400 dilution or higher in the tube method.

Cross Agglutination Reaction Between Brucella and Proteus.—Observations have been made with cross agglutination in patients suffering from tularemia or brucellosis, but such reactions had not been noted until Calder[1] called attention to this phenomenon between Brucella and Proteus. He found significantly high agglutinin titers for Proteus in chronically febrile patients who gave no clinical story or physical signs suggesting the possibility of typhus or Rocky Mountain spotted fever. A detailed study was made by the Brucellosis Laboratory of the Clayton Foundation for Research, at Houston, Texas, using six organisms as follows: *Brucella abortus; Brucella melitensis; Pasteurella tularensis; Proteus OX19,* from the National Institutes of Health; *Proteus OX19,* obtained from the laboratories of the Texas State Department of Health; and *Proteus HX19* strain, prepared by treating a motile strain with 0.4 per cent Formalin. The macroscopic method was used. Preparations were read against a strong light in a darkened room after two hours' incubation at 37° C., 22 hours in a refrigerator, and 24 hours at room temperature.

As a result of their work, it was found that agglutinins for *Pasteurella tularensis* were encountered very seldom, and when present, the titer was usually low. Chronically ill patients, therefore, are not likely to show tularensis agglutinins, which would lead to erroneous diagnoses. On the other hand, agglutinins for Proteus were frequently noted in these ill persons. The titers were at times high enough to be interpreted as positive Weil-Felix reactions. The explanation appears to be that there is an antigenic relationship between Brucella and Proteus, probably due to certain components shared by the respective somatic antigens.

The following warning, therefore, must be issued in doing laboratory work on chronically ill persons—a high Proteus agglutination must be very carefully studied before it is taken as an indication in a chronically ill patient as a truly positive Weil-Felix reaction. On the contrary, a very high titer showing Proteus agglutinins exceeding the Brucella agglutinins in brucellosis should arouse the suspicion that the patient is suffering from brucellosis and not from typhus or Rocky Mountain spotted fever.

Practically, these reactions should not be confusing if repeated tests are made, for, contrary to the ascending titers seen in typhus, the Proteus agglutinins do not rise in brucellosis with the passage of time.

Diagnosis by Complement Fixation Tests.—(See pages 1742 ff.)

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[1] J. Bact. **41**: 593, 1941.

ANIMAL TESTS

For the diagnosis of typhus, the test is carried out by inoculating susceptible animals with whole blood or a fresh saline suspension of the macerated insect vector subcutaneously or intraperitoneally. Susceptible animals are guinea pigs, rabbits, and monkeys. At the end of a week and not later than two weeks, in guinea pigs, we note a fever which lasts from three to fourteen days. The virus is found in the blood but is more concentrated in the brain. It can be maintained in a virulent state by passage. Animals recover and are then immune to reinfection. The female shows only fever but the male guinea pig develops characteristic inflammatory and hemorrhagic lesions of the genitals. The virus of endemic typhus causes a marked swelling of the scrotum while that of epidemic typhus causes little or no visible change. Rickettsiae may be found in the tissues, especially in the scrapings from the tunica vaginalis.

Rabbits and monkeys are also susceptible to infection but are more resistant than guinea pigs. They do not show symptoms but give a positive Weil-Felix reaction whereas the blood serum of infected guinea pigs gives a negative reaction.

CULTURE METHOD

The chorio-allantoic membrane is an excellent medium for culturing Rickettsiae and may be used in place of many of the animal tests, if necessary. Egg inoculation is given on pages 1473 and 1745.

Rocky Mountain Spotted Fever.—This disease has been reported from western states, particularly Montana and Idaho. It was first noted in the Snake River Valley of Idaho, about 1893, and in the Bitter Root Valley of Montana.

Diagnosis of Rocky Mountain Spotted Fever by Animal Tests.—Inject 1 c.c. of patient's blood intraperitoneally into male guinea pigs. The animal develops fever. Lesions noted are characterized by erythema and swelling of the scrotum, with appearance of small red macules. Ulceration occurs. Testicles are enlarged and surrounding tissues are edematous. Spleen is enlarged. As noted above, most of the animals give a positive Weil-Felix reaction and a low titer. Monkeys are less susceptible. Complement fixing antibodies appear soon.

Maxcy[1] affirms the usefulness of the rabbit test in the diagnosis of Rocky Mountain spotted fever. Specific agglutination of *Proteus OX19* by the serum of rabbits two or three weeks after inoculation with Old World typhus was reported by Weil and Felix[2] in 1920. Foot[3] had previously shown that rabbits were likewise susceptible to infection with passage virus of the Rocky Mountain spotted fever. Munter[4] had no difficulty in demonstrating agglutinins for *Proteus X19* in the serum of rabbits inoculated with a passage strain of Rocky Mountain spotted fever virus. Maxcy, using strains received originally from A. Felix, repeatedly plated the cultures for purity of the ''O'' or nonmotile variant and absence of ''H,'' or motile, spreading colonies before the final growth on infusion agar in Blake bottles. According to Felix, the purity of the ''O'' phase is a more important consideration than the question of whether living or killed organisms are used as antigens. Rabbits inoculated with blood from Rocky Mountain spotted fever cases develop agglutination for the *Proteus X19*. The method used by Maxcy to carry out the test is to give 3 c.c. of blood intravenously and 3 c.c. intraperitoneally. These rabbits showed, between the 4th and the 9th day of the disease, distinct agglutination with *Proteus X19*.

The procedure, therefore, of proving the diagnosis of Rocky Mountain spotted fever is to inoculate rabbits directly with citrated blood and carry out agglutination tests.

RABIES

Rabies is a very common disease. It is known as rabies, lyssa, tollwut, hundswut, hydrophobia, rage, rabbia, canine madness. It is an acute, fatal, infectious disease, and is found principally in animals of the canine variety, such as dogs, wolves, foxes, hyenas, jackals. Its manifestations denote great irritation of the central nervous system, followed by definite symptoms of paralysis, after a variable period of incubation. There are very few pathologic anatomic findings visible to the naked eye in lower animals and human beings dying with this disease. The disease is commonly communicated from sick animals to man by biting, the saliva supposedly carrying the virus of this disease.

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[1] J. Infect. Dis. 58: 288, 1936.
[2] Wien. klin. Wchnschr. 33: 423, 1920.
[3] J. Med. Research 39: 495, 1919.
[4] Ztschr. f. Hyg. u. Infektionskr. 109: 124, 1928.

Rabies is due to a virus. Attempts to cultivate the street virus of rabies have been successful as reported by Kanazawa,[1, 2] who used a medium consisting of rabbit embryo brain tissue suspended in Tyrode solution, without serum; by Webster and Clow[3, 4] in a medium consisting of mouse and chick embryo brain tissue suspended in serum Tyrode; and by Schultz and Williams,[5] who confirmed the latter's work. Direct in vitro cultivation of the street virus of rabies was not reported by these investigators. However, Plotz and Reagan[6] report in vitro cultivation of street virus.

The virus is maintained, however, either in animal brains or in developing chick embryos.

In 1903, Negri described characteristic bodies, small round corpuscles which were found in the ganglion cells of the sick animals. He believed these were protozoa and thought that he had found the cause of the disease. It has since been proved that these are not protozoa and therefore are not the causative factors of the disease, although they are present constantly in about 90 per cent of animals and human beings dying with rabies.

Microscopic diagnosis of rabies can therefore be made by finding Negri bodies in the large cells of the cornu ammonis of the brains of animals submitted to the laboratory for examination. The laboratory is sometimes consulted before the dog is actually captured and killed. Our policy, in case of a dog bite, is to observe the dog and look for symptoms of rabies rather than to kill him precipitantly and not find the Negri bodies; in other words, a policy of watchful waiting, of observation of the dog, and noting the presence or absence of suspicious symptoms. The history of the dog should be taken into account in ascertaining the probable diagnosis of rabies. History shows that dogs incubating rabies frequently change their habits. They leave home, wander about, eat foreign bodies, such as pieces of wood, sticks, pieces of iron. When the disease is fully developed, their behavior is very characteristic. They run along snapping and biting at everything that interferes with their progress. There soon develops muscle paralysis, followed by excessive salivation and convulsions. The bark becomes hoarse or disappears, the lower jaw hangs and the tongue may protrude. The hind legs become weak, ataxia develops and the legs no longer support the dog. The entire duration of sickness in the dog before death ensues is from four to seven days, occasionally

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[1] Jap. J. Exper. Med. **14**: 519, 1936.
[2] Jap. J. Exper. Med. **15**: 17, 1937.
[3] Science **84**: 487, 1936.
[4] J. Exper. Med. **66**: 125, 1937.
[5] Proc. Soc. Exper. Biol. & Med. **37**: 372, 1937.
[6] Science **95**: 102, 1942.

longer. They are likely to bite man, live stock, trees, fences. This is what is known as "furious rabies." There is another type known as "paralytic rabies" in which the dogs do not bite but simply have the symptoms of difficulty in swallowing. Frequently the owner thinks his dog has a foreign body in his esophagus. Do not put the bare hand into the mouth of a dog with these symptoms in order to remove the supposed foreign body; there is a great possibility of infection from the saliva. If the animal must be killed, it is advisable to tell the person in charge of the investigation for rabies in a dog to be sure when killing the dog not to injure the brain. The dog should preferably be shot through the heart, or other part of the body, not through the head.

One must remember that diseases other than rabies may produce some of the symptoms of rabies infection in dogs, so that the clinical diagnosis in dogs is not always easy. Distemper may produce excessive salivation, convulsions, and leg weakness. "Blacktongue" has been at times confused with rabies. Many other conditions may induce irritability, convulsions, and muscle paralysis in dogs.

In general, the best policy to pursue with a dog suspected to be rabic is to confine him, keep him alive under the eye of an experienced veterinarian, and kill only when the characteristic symptoms seem to indicate beyond reasonable doubt that rabies exists.

Rabies Infection in Man.—It is often ushered in by tingling sensations and soreness, especially in the region of the bite. There follow loss of appetite, restlessness, irritability, and melancholic symptoms. Headache and emaciation follow. The stage of excitement starts abruptly, with general excitement and chorea-like symptoms. Intense spasms set in, with difficult and painful respiration. The onset of convulsions of the pharyngeal muscles gives the disease its name "hydrophobia" which is really not fear of water, but convulsive efforts when the patient attempts to drink or eat. The convulsions which have been seen by this author in patients with rabies were so severe that the patients could hardly be restrained from falling out of bed. Hallucinations of sight and hearing soon begin. Excessive sweating and salivation occur and swallowing becomes impossible. Paralyses begin, often in the muscles adjacent to the site of the bite. The pupils dilate, reflexes disappear, and death occurs, usually in from one to eight days after the first symptoms started.

There has been considerable discussion as to the incubation period of this disease in man: the author has a record of one case, a bite on the face in a boy of fourteen years. In this case, the diagnosis in the biting dog was made immediately after the attack on the boy took place; anti-rabic serum was started, the boy developed symptoms of rabies on the sixth day after the bite. These symptoms were ushered in by a change in the expressions of the eyes—unusual brightness and fixation of the eyes, followed by a rise in temperature, then convulsions and death within forty-eight hours. Diagnosis in the boy was confirmed by autopsy finding of the Negri bodies in the brain and production of rabies in a rabbit after ten days. The author has a record of one patient of an incubation period one and one-half years after receiving a bite. The literature speaks of two years as the longest period of incubation.

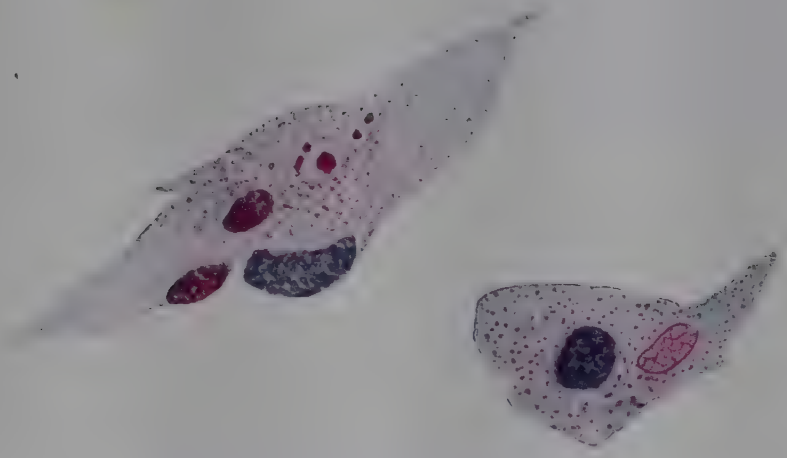

PLATE XLV.—NEGRI BODIES, VAN GIESON STAIN.

Technic of the Test for Negri Bodies

The dog's head is amputated and brought to the laboratory as soon as possible before decomposition sets in. In summer it is advisable to pack the head in ice during transportation. The operator should wear rubber gloves. The head is placed upon a board and the skull cap is removed by sawing. The brain is removed from the skull. An incision is made straight down on one side of the hemisphere into the lateral ventricle. At the posterior part of the floor of the lateral ventricle, an incision is made deep down into the cortex. Here is found a structure known as "Ammon's horn," where Negri bodies are most likely to be found.

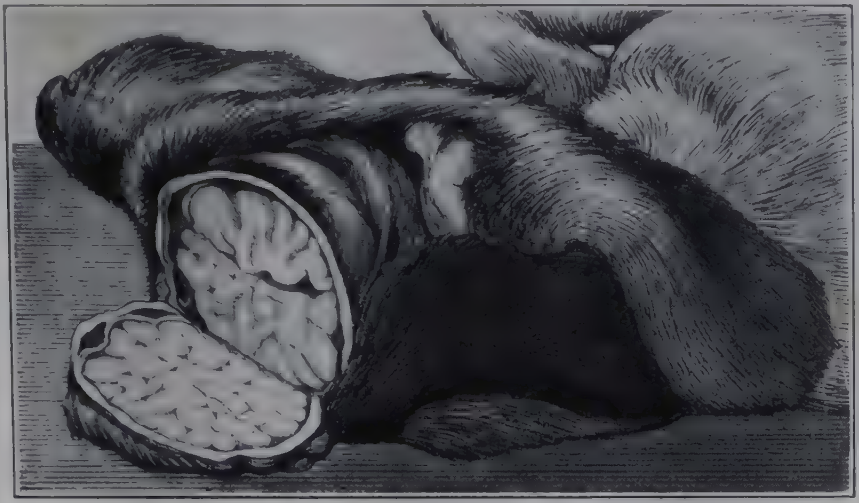

Fig. 406.—Section through dog's head for Negri bodies examination.

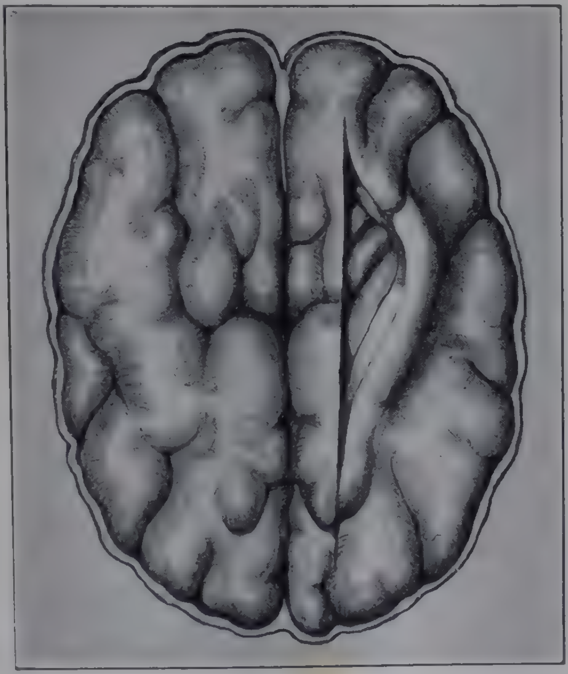

Fig. 407.—Dog's brain, showing method of locating Ammon's horn.

To make an impression preparation, cut out a 1 to 2 mm. cross section from the middle of Ammon's horn and place it on a piece of blotting paper.

Use a clean slide and make several impressions by pressing the slide down on the cut surface of the section.

While still wet, layer over with Seller's stain for 10 seconds.

Rinse with tap water, and allow to dry.

Examine with an oil immersion objective, or mount with a cover slip, using Canada balsam or gum damar.

Another method is to place a small piece of the brain, about a quarter of an inch square, in a Petri dish, using sterile forceps.

Remove a piece of tissue and place on a glass slide.

Superimpose another slide upon it and make smears by pulling the slides against each other in opposite directions. This gives two smears at the same time.

Similar smears should also be taken from the cerebellum and from various parts of the cerebral cortex.

Demonstration of Negri Bodies

Seller's Stain

Stock Solution:

(a) Saturated solution of basic fuchsin in methyl alcohol
(b) Saturated solution of methylene blue in methyl alcohol

Staining Solution:

Stock basic fuchsin	3.5 c.c.
Stock methylene blue	15.0 c.c.
Methyl alcohol	25.0 c.c.

The staining solution may be kept in a dropping bottle. If stored in a refrigerator when not in use, it may be used for several months.

Negri bodies appear mauve to pink-red; the basophilic staining inner bodies are well demonstrated. The cell cytoplasm, nuclei, and nucleoli stain blue. Interstitial tissue and fibrillae stain pink.

If the preparations are from human material, there is little chance of confusing the inclusion bodies of rabies with those which occur in other diseases. In dog brains, however, other inclusion bodies such as those found in canine distemper may be found. These inclusion bodies are pale red and more refractile than those due to rabies and occur more frequently in the thalamus and lentiform nuclei than in the Ammon's horn.

Atypical intracytoplasmic inclusion bodies are sometimes found in the brains of nonrabid mice; these are probably due to a natural virus disease of mice. Such inclusions stain pink to bright red, are refractile, uniformly round, and do not have any inner structure; they show a blue-staining margin.

Description of Negri Bodies

Negri bodies are sharply outlined, round, oval, or somewhat irregular structures of variable size, from 1 to 27 microns in diameter. In dogs the average diameter is from 4 to 10 microns. Negri divides them according to size as follows:

Smaller forms from 1 to 5 microns in diameter.
Medium forms from 10 to 15 microns in diameter.
Large forms, 22 to 23 microns in diameter.
Very large forms, 27 microns.

They consist of a hyaline-like cytoplasm, in which when properly stained, one or more chromatin bodies can usually be seen. They are situated chiefly within the cytoplasm of the large cells of the central nervous system, as already stated. In many cases they suggest red blood cells lying within nerve cells.

Animal Inoculation.—If Negri bodies are found, this is sufficient evidence for the diagnosis of rabies. If they are not found, it is necessary to inoculate animals. The favorite animals in the past have been guinea pigs and rabbits. Since the demonstration that the intracerebral injection of rabies virus into white mice produces a typical and constant infection, the white mouse has become increasingly popular as a test animal.[1] The chief advantages of the mouse are the low cost, making it possible to use several animals for one specimen; the relatively short incubation period, ordinarily six to thirteen days with street virus; and the consistency of production of inclusion bodies in the brains of mice infected by intracerebral injection.

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[1] William A. Hagan, et al.: Ann. New York Acad. Sc. 48: 373, 378-381, 1947.

A small amount of the hypocampus is rubbed up, with sterile precautions, in a sterile mortar with sterile physiologic saline solution. This is picked up in a sterile 1 c.c. or 2 c.c. syringe, and 0.03 c.c. is injected into the animal, just behind the lateral corner of the eye, with a short bevel 24 gauge needle.

Incubation in guinea pigs is eight to twenty days. Incubation in rabbits is from fourteen to twenty-one days. The onset of the disease in each animal is marked by paralysis of the fore or hind quarters. Diagnosis can be confirmed by finding Negri bodies in the brain of the inoculated animal.

It is to be emphasized that the failure to find Negri bodies is not proof of the nonexistence of rabies. In all cases, the history of the animal, the circumstances of the bite, and, last of all, the inoculation into guinea pigs and rabbits of brain matter from the suspected dog must be carried out. In cases in which the bite has been inflicted and the dog has escaped and there is no way of telling from the examination of material of the dog whether he has or has not rabies, the safest course to pursue is to use the Pasteur antirabic virus as a preventive measure.

The Pasteur Treatment for Rabies

Johnson[1] advises the following **criteria for vaccine treatment.** He states that a number of factors must be considered before advising rabies vaccine treatment for persons bitten or scratched by domestic and wild animals. It should be ascertained whether there has been any rabies reported in the vicinity during the previous year and also whether the animal was apprehended or killed. If the animal was captured, it should be turned over to a veterinarian, who should be consulted as to whether or not there are clinical signs of rabies. Clinically normal animals should be quarantined for 14 days. If the animal subsequently shows clinical signs of rabies, the attending physician should be notified immediately. If the animal is killed or dies while in quarantine, the brain should be examined for Negri bodies and, if negative, animal inoculation should be made.

If the patient has been bitten by a stray dog which has not been apprehended or which was killed immediately, an attempt must be made to ascertain the animal's behavior before biting the patient. An attempt should be made to locate the animal.

Rabies vaccine treatment must be begun immediately when a person has been bitten or scratched under the following circumstances:

1. The animal was apprehended and presented clinical signs of rabies.
2. The animal was killed and the brain found positive for rabies by microscopic examination.
3. The animal was killed, the brain was negative, but the animal was suspected of being rabid.
4. The person was exposed by a stray animal which escaped and where rabies was known to be present in the community.

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[1] Johnson, Harald N.: Rabies, New York, 1943, The Oxford University Press.

Vaccine treatment is recommended also for persons who have handled animals diagnosed as rabic by clinical or laboratory examinations, or when fresh abrasions of the skin have been contaminated by the saliva of the infected animal.

When an animal is captured after having bitten a person, the animal must not be killed, but should be confined under the supervision of a veterinarian. If the animal is rabid, it will usually die within 2 or 3 days. Since the inclusion bodies of rabies are often absent in the early stages of the disease, it is important that the animal not be killed immediately upon biting.

Even though the animal was apparently normal at the time of biting, treatment should be instituted if the animal later develops clinical signs of rabies while in quarantine. However, if the biting dog remains well for 10 days while held in quarantine, treatment may be discontinued.

When an animal is killed immediately after biting, and the brain is examined and found negative by microscopic examination, a confirmatory mouse inoculation test should be performed.

Johnson advises the following **local treatment** of persons bitten by or exposed to the saliva of rabid animals. It has been shown that infection can be produced readily in animals by rubbing the virus into the scarified skin. Wounds in areas heavily supplied with sensory organs, such as the face and hands, are especially dangerous. Wounds which penetrate the muscle tissue probably are even more serious.

Shaughnessy and Zichis[2] compared the effectiveness of 20 per cent green soap solution, nitric acid, and tincture of iodine for treatment of wounds in experimentally infected guinea pigs, and showed that the green soap solution is as effective as nitric acid cauterization where the treatment is carried out within 2 hours after exposure. Tincture of iodine is less effective.

Harris[3] recommends carbolic acid cauterization instead of nitric acid, as he has found it more effective. The first and most important step in treatment of wounds produced by supposedly rabid animals is cleansing with warm water and soap to remove the saliva and to encourage bleeding before cautery is applied. Deep lacerations can be effectively cleansed with a syringe.

All patients should receive local treatment with carbolic acid. The wound should be sponged dry and the carbolic acid applied with a capillary dropper or wooden applicator, taking care not to miss small or superficial abrasions. The margins of torn skin of larger wounds must receive particular attention. If the wounds are severe, cauterization may be performed under anesthesia. If the lacerations occur on the face, thorough cleansing should be carried out with green soap solution, followed by carbolic acid cauterization of superficial abrasions, puncture wounds, and the margins of the lacerated skin.

Harris' method of local treatment is carried out as follows: The wound should be mechanically cleansed, cauterized with pure carbolic acid, and immediately bathed with 95 per cent alcohol.

The next and most important step is to determine whether or not the biting animal is rabid, for if it is, vaccine treatment must be begun at once.

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[2] Shaughnessy, H. J., and Zichis, J.: Proc. U. S. Livestock San. Assoc., pp. 1-4, 1941.
[3] Harris, D. L.: Personal communication.

Harris states that any biting animal should be suspected of being rabid until proved otherwise. If the animal cannot be confined and observed, it should be regarded as rabid.

No preparation of antirabies vaccine is invariably effective or entirely free from dangerous sequelae. In rare cases, the injection is followed by a severe and sometimes fatal paralysis. This is probably due to individual sensitiveness to rabbit's brain and cord and is probably not the result of an infection by the vaccine.

Recommendations for the Control of Rabies in the United States

These recommendations are well set forth by Hagan and associates.[1]

Rabies control should be coordinated through some national agency. This control work is strictly a veterinary problem. Hagan recommends that the U. S. Department of Agriculture, Bureau of Animal Industry, be given authority to coordinate and supervise rabies control work. This is not possible under existing Federal legislation. A bill has been introduced in Congress which would include the dog and other domestic carnivores with those domestic animals subject to the supervision of this branch of our government. If this bill is passed a nationwide rabies control program could be developed through each State Department of Agriculture, Live Stock Sanitary Division. At the present time, rabies control work is conducted on a municipal or county basis under the authority of the City or County Board of Health, and the work is maintained only as long as the emergency persists. Hagan states further that rabies control work on a state basis should be under the supervision of a full-time veterinarian. Rabies in animals should be made a reportable disease, new cases to be reported immediately to the veterinarian in charge of rabies control in the State, and weekly, through regular reporting channels, to the U. S. Department of Agriculture, Bureau of Animal Industry. Past experience has shown that the disease is apt to be of epizootic proportions in a given locality, before the State authorities responsible for rabies control are aware of the outbreak. The heads of all domestic and wild animals suspected of having rabies should be submitted for examination, regardless of whether or not there was human exposure to the disease. Rabies existing in a wild life species should be reported to the U. S. Fish and Wild Life Service, as well as to the State Wild Life Department, so that a cooperative control program may be developed.

Taxation and licensing of dogs is further recommended as the most effective means for insuring enforcement of rabies control regulations. The traditional rabies control program is one which requires strict quarantine for all dogs for a period of six months after the last reported case of rabies. Field experience has shown that vaccination of all owned dogs, carried on in conjunction with collecting and impounding of unvaccinated dogs found at large, has brought about the elimination of rabies. This method of rabies control has been used effectively in several foreign countries, as well as in many communities in the United States. Compulsory vaccination of dogs for rabies, on a State-wide basis, has been difficult to enforce. In Alabama, the enforcement of vaccination of dogs for rabies, combined with collection of unvaccinated dogs found on the street, was effective in limiting the disease.

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[1] Ann. New York Acad. Sc. 48: 373, 378-381, 1947.

Strict quarantine for all dogs should be enforced for a period of at least thirty days, as soon as rabies appears in a community. Then dog owners should be required to have their pets vaccinated or keep them confined until the area is officially certified free of rabies. Vaccinated dogs, properly tagged, may be allowed at large thirty days after vaccination. A course of three weekly injections of vaccine will produce a more certain immunity to rabies. Dogs known to have been exposed to rabies must be destroyed, or kept confined for six months.

Hagan emphasizes that unvaccinated dogs and stray dogs should be picked up promptly throughout the control program. This should be continued for at least ninety days following the last reported case of rabies.

The statistics on rabies for the United States show that domestic cats are infected quite frequently. The bite of a rabid cat is probably more dangerous than that of a dog. Cat owners should have their pets vaccinated by the three-injection method. Stray cats should be impounded and destroyed. If rabies becomes established among wild animals, it is necessary to carry out a program of reduction of the number of the affected species until the disease disappears.

DEMONSTRATION OF INFLUENZA VIRUS IN THROAT WASHINGS

The detection of influenza virus in throat washings is an important method, during epidemics, of determining the causative virus as rapidly as possible. Various methods have been described; for instance, isolation of influenza in ferrets and mice,[1] in hamsters,[2] and in chick embryos by amniotic[3] or allantoic[4] inoculation. Egg inoculation, with subsequent Hirst test, is used in actual practice. Refer to Egg Inoculation.

Technic.—

Inoculate 6 to 24 chick embryos 11 or 12 days of age by the amniotic route with each throat washing filtered through Berkefeld N filters. With most specimens the window method of Burnet[3] is used, but with some the stab method described by Hirst[5] may also be employed. Carry out three to five amniotic passages at intervals of two to five days with each throat washing before the result is considered negative. Virus may be detected in the amniotic or allantoic fluids by agglutination of chicken erythrocytes[6] and may be identified by inhibition tests with known types A and B antisera.

Allantoic inoculations are made with unfiltered throat washings centrifuged for 20 minutes at 4,000 r.p.m. in an angle head and also with duplicate specimens treated with Zephiran*[7] at a dilution of 1:20,000 for 20 to 30 minutes. Detect and identify the virus by the method used for the amniotic passages. Withdraw allantoic fluids from the third to the sixth day after inoculation daily by a needle and syringe from the living embryos and test for agglutinins so that they may be harvested or passed if positive.

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*Zephiran, manufactured by Alba Pharmaceutical Co., Inc., is a mixture of high molecular alkyl dimethyl benzyl ammonium chlorides.

[1] (a) Brit. J. Exper. Path. 16: 566, 1935; (b) Proc. Soc. Exper. Biol. & Med. 32: 1172, 1935.

[2] Proc. Soc. Exper. Biol. & Med. 49: 105, 1942.

[3] Brit. J. Exper. Path. 21: 147, 1940.

[4] Science 98: 516, 1943.

[5] J. Immunol. 45: 293, 1942.

[6] Australian J. Exper. Biol. & Med. Sc. 21: 55, 1943.

[7] Science 96: 543, 1942.

ETIOLOGICAL AGENT OF INFECTIOUS HEPATITIS

Numerous reports have appeared in the literature describing the occurrence of jaundice in man following the administration of human blood products and indicating that the icterogenic agent or agents of such homologous serum jaundice[1-4] and post-vaccinal jaundice[5-10] are filtrable through a Seitz filter and can withstand heating to 56 to 60° C. for 30-60 minutes. More recently, certain planned experiments,[11, 12, 13] on the transmission of these diseases to human volunteers have corroborated these facts. A most important report on the properties of the icterogenic agent of the naturally occurring disease, infectious hepatitis, appeared in 1945.[14] The agent of this disease is a virus.

As in homologous serum jaundice and post-vaccinal jaundice the icterogenic agent of infectious hepatitis is filtrable and withstands heating to 56° C. for 30 minutes. It also demonstrated that this agent can be transmitted in serial passage in human volunteers.

There is plenty of evidence that during World War II transfusions were the commonest cause of jaundice among military forces.[15] A large amount of plasma was collected during World War II by the American Red Cross for use of the Services. Necessarily, in this large amount of plasma, undoubtedly some bloods were taken from recovered cases of hepatitis. Even when diluted with the pool with which this plasma was placed, there was sufficient substance present to cause trouble. A tremendous amount of this plasma was left after the war and is being distributed to the hospitals. The problem of whether or not this plasma will induce infectious hepatitis is serious enough to be considered. The danger is proportional to the number of donors who supply plasma. It has been proved that the virus of this disease may be present in the blood for as long as 60 days prior to the onset of jaundice.[16] In view of this fact, blood banks must adopt some procedure for the observation of donors over a period of time both prior and subsequent to their actual use. This will unquestionably reduce introduction of contaminated bloods into the pool. The use of human immune gamma globulin has been suggested as an agent to be added to pooled plasma prior to its use. It might be administered prophylactically early in the incubation period to all persons who have received plasma.[15]

It is generally believed that, from a laboratory standpoint, the differentiation of viral hepatitis from obstructive jaundice can be made by two tests.

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[1] Brit. M. J. **1**: 750, 1943.
[2] Lancet **1**: 83, 1944.
[3] Lancet **1**: 814, 1944.
[4] Unpublished results: Paul, J. R., Havens, W. P., Jr., Philip, C. B., and Sabin, A. B.
[5] Tr. Roy. Soc. Trop. Med. & Hyg. **31**: 279, 1937.
[6] Proc. Roy. Soc. Med. **31**: 799, 1938.
[7] Am. J. Trop. Med. **18**: 111, 1938.
[8] Tr. Roy. Soc. Trop. Med. & Hyg. **32**: 575, 1939.
[9] Lancet **2**: 301, 1944.
[10] Am. J. Hyg. **39**: 337; **40**: 35, 1944.
[11] Proc. Soc. Exper. Biol. & Med. **57**: 206, 1944.
[12] Pub. Health Rep. **58**: 1233, 1943.
[13] Lancet **1**: 622, 1944.
[14] Proc. Soc. Exper. Biol. & Med. **58**: 203, 1945.
[15] J. A. M. A. **128**: 932, 1945.
[16] J. A. M. A. **128**: 911, 1945.

The cephalin flocculation test is generally increased in viral hepatitis and the alkaline phosphatase test tends to remain normal in this condition.

Patients in the hospitals are exposed to hepatitis through the use of nonsterile or poorly cleansed syringes, needles, or surgical instruments, and through the injection of blood or blood products, such as plasma, which may contain the virus of hepatitis. This type of hepatitis is distinct from epidemic hepatitis. The incidence of serum hepatitis following the use of pooled plasma is high. The risk is greater whenever a large number of donors are used in the pool. The danger of hepatitis following the use of blood alone is rather slight. Acceptable figures indicate that serum hepatitis develops in 2.5 to 7.3 per cent of the recipients of pooled plasma, while less than 1 per cent develop this disease following use of blood transfusions alone. Plasma has been irradiated with ultraviolet light in an attempt to inactivate the virus of hepatitis.

Sborov, Giges, and Mann[1] reported on the incidence of hepatitis in a large number of recipients of pooled plasma in three army hospitals. They found in a study of 587 wounded soldiers that 21.9 per cent of those who received both plasma and blood developed hepatitis. Their study also indicated that, following the use of plasma irradiated with ultraviolet light, the incidence of hepatitis was no different from that following the use of nonirradiated plasma. The mean incubation period of hepatitis was about 90 days, thus indicating that the hepatitis indeed represented the serum type.

Pooled human plasma in a stockpile for use by civilian physicians in hospitals carries with it the risk of hepatitis. Furthermore, even those batches of pooled plasma which are irradiated with ultraviolet light are just as likely to cause hepatitis as the nonirradiated variety.

LYMPHOGRANULOMA VENEREUM*

Lymphogranuloma venereum has also been known as "climatic bubo," the sixth venereal disease, Nicolas-Favrè disease, subacute inguinal lymphogranulomatosis, lymphogranuloma inguinale, and by many other names. It is a generalized disease acquired, like syphilis, usually by sexual contact. It is principally genitorectal, with preference for lymphatic and nervous tissues, with possible invasion of other organs.

It is caused by a virus which is filtrable through Chamberlain 13 and Berkefeld V filters. Dimensions vary from a fraction of a micron to 4 to 6 microns, exceptionally even larger forms being found.

It has not been cultivated on routine laboratory media but has been transmitted to animals.

Rake, McKee, and Shaffer[2] noted similarities and differences between infectious agents of the "lymphogranuloma-psittacosis" group, which includes agents of lymphogranuloma venereum, trachoma, inclusion conjunctivitis or blennorrhea, psittacosis, and human atypical pneumonia (virus pneumonia), acute meningopneumonitis, and rat pneumonitis.

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*After Soto Rozas in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, St. Louis, 1948, The C. V. Mosby Co.

[1] Arch. Int. Med., Nov., 1953.

[2] Proc. Soc. Exper. Biol. & Med. 43: 332, 1940.

The agent is susceptible to the action of sulfa drugs. It can be preserved in mouse brain tissue or in chick embryo in the frozen state. It resists desiccation, pure glycerin, and freezing under vacuum, but does not resist ultraviolet light, 0.1 per cent Formalin, 2 per cent urea, 50 per cent glycerin, or ether.

The Frei test has been used successfully in making this diagnosis.

The Frei Reaction

This is a method of making the diagnosis of this disease by the use of an antigen prepared from the pus withdrawn by aspiration from the bubo. It was published by Frei[1] in 1925. The value and specificity of the test have been demonstrated by Frei in subsequent papers[2] in 1927 and 1932, and have been confirmed by Frei and Hoffmann[3] in 1927, and by a number of other workers.

Frei's directions for the preparation of this antigen are as follows: The person from whom the antigen is to be made should be proved free from tuberculosis and venereal diseases by clinical examination and negative tests for syphilis, gonorrhea, and soft chancre. The pus must be withdrawn from a gland that has undergone softening but not fistulation. Aspirate by means of syringe and thick needle, under sterile precautions, obtaining as far as possible, blood-free pus. This pus is mixed with physiological saline in the proportion of 1 part of pus to 5 parts of saline in a sterile tube, and immediately put up in 0.5 to 1 c.c. doses in hard-glass ampules. It is heated to 60° C. for two hours over a water-bath, and the following day at 60° C. for one hour. This material is tested for sterility by culture under aerobic and anaerobic conditions at room and incubator temperatures. This antigen should give a negative reaction in the skin of normal individuals. The antigen should be preserved at a low temperature and unexposed to light. If an ampule is opened, the remaining contents should be thrown away or resterilized.

Method of carrying out the test: Disinfect the skin with alcohol. Use the skin of the outer forearm. Inject intracutaneously 0.1 c.c. antigen. A corresponding area on the other side of the body should be injected with the same amount of normal saline. It is well to inject two normal controls with the same antigen and the same saline in the same way and at the same time. Read the result after forty-eight hours, and not before. A positive reaction consists in the production of an infiltrated inflammatory dome-shaped area one-half cm. in diameter at least, and often larger, which can be felt as well as seen. It is often noted that there is a small area of necrosis in the center surrounded by a red zone.

Antigens usually remain active for several months, sometimes for a year, but not always.

The Vesicular Test for Lymphogranuloma Venereum (Inguinale)

Owing to the difficulty of obtaining material at times for the making of the original Frei antigen, Ottolina[4] has suggested the use of concentrated spinal fluid from a known case, using this material for skin inoculation. This is based upon the fact that in experimental infections of this disease, the cerebromedullar axis is prevailingly affected. Spinal fluid, therefore, constitutes a human antigen, easy to obtain, aseptic, and with a small amount of albumin.

The method described by Ottolina is to perform a spinal puncture under aseptic conditions and carry out a vacuum concentration of the fluid. By reducing 10 c.c. of fluid to 1.5 c.c., a colloidal-like solution is obtained which is opalescent and foams upon agitation.

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[1] Klin. Wchnschr. 4: 2148, 1925.

[2] Klin. Wchnschr. 6: 2042, 1927; Klin. Wchnschr. 11: 512, 1932; Dermat. Wchnschr. 95: 1576, 1932.

[3] Arch. f. Dermat. u. Syph. 153: 179, 1927.

[4] Am. J. Trop. Med. 21: 597, 1941.

If this concentrated fluid is injected intradermally a vesicle appears twenty-four hours later at the point of injection; this phenomenon has been called the "vesicular test." The vesicle may be ovoid, circular, or triangular but there is a definite trend toward the hemispheric form. The ovoid type measures 4 mm. in length, 2 mm. in width, and 1 mm. in height while the more rounded forms approach approximately 3 mm. by 2 mm. by 1. The color of the skin covering the blister is light or dark brown, sometimes dark gray. The blistered skin always has a darker shade than the surrounding skin.

Twenty-four hours after the injection the blister preserves the same morphology, the fluid within showing a light tension. Forty-eight hours later a certain umbilication may be noted, the fluid tension has relaxed, and the blister skin is more colored than on the first day. Three days later there is a distinct flaccidity in the blister and desiccation begins at that time and ends in between four and five days. Five or six days after its appearance the blister is only a pigmented spot about 2 mm. in diameter. The vesicular fluid has a light brown color and microscopically shows a few polynuclear neutrophilic and mononuclear leukocytes. This fluid is sterile.

Technic is to concentrate fluid employed from 10 c.c. to 2 c.c. and inject intradermally 0.3 c.c.

It is interesting to note, according to Ottolina's researches, that this test, when applied to the positive patient from whom it was obtained, produced a typical positive reaction. In other words, it is an autoreaction, which emphasizes its diagnostic value.

Mouse Brain Antigen for the Frei Reaction

Grace and Suskind

Hellerström[1] has shown that antigens made from the brains of monkeys infected through subdural injection give the same specific Frei reaction when used on human beings as when the human antigen is used. Working along this line, Grace and Suskind[2] developed a mouse brain antigen which is more highly potent than that made directly from the same human material used in the preparation of the brain antigen. This antigen is a 10% emulsion of infected mouse brain; for each test 0.1 c.c. is injected intradermally, using a 1 c.c. tuberculin type of syringe with a 27 gauge needle.* If positive, the raised papular reaction is noted in from forty-eight to seventy-two hours. If negative, no reaction occurs; if one uses an injection of a similar emulsion of normal mouse brain as a control, no reaction appears.

Chick Embryo Antigen for the Frei Reaction

The three sources of antigen for the Frei test just described are: (a) pus from the inguinal bubo of the patient, (b) macerated material from diseased glands, and (c) emulsions of the brains of mice intracerebrally infected with virus. Since antigens prepared from human pus are not readily available, and since considerable doubt has been expressed as to the reliability of the mouse brain antigen, a successful method of producing antigen has been evolved by propagating the virus of lymphogranuloma venereum on the chorio-allantois of developing chicks. This has been successfully carried out by Rake, McKee, and Shaffer[3] working in the Squibb Institute of Medical Research. These workers developed a method for obtaining a highly potent and purified antigen for this test. The virus was inoculated into the yolk-sac of 5- or 6-day-old fertile eggs, as demonstrated by Fig. 408.†

By suitable methods of purification a virus was obtained which, when undiluted, was about one million times more potent than the lymphogranuloma virus obtained from mouse brain. Not only did the yolk-sac method yield a more potent virus than the other methods but, also, by a process of differential centrifugation, a practically pure suspension of the virus could be obtained. In other words, the extraneous inert material from the yolk-sac can be almost completely eliminated.

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*The Lederle Laboratories have put this on the market in syringes ready for use. They furnish it in 1 c.c. syringes for one dose and in 10 c.c. syringes for ten doses. There is an expiration date on these packages.

†Furnished by permission of E. R. Squibb & Sons.

[1] Acta dermat. venereol., supp. 1, pp. 5-244, 1929.

[2] Proc. Soc. Exper. Biol. & Med. 32: 71, 1934.

[3] Proc. Soc. Exper. Biol. & Med. 43: 332, 1940.

In a very interesting trial of this material, Sulkin, Fletcher, Huber, and Reh* reported forty-two patients with active lymphogranulomatous infection or with a history of past infection, who were tested with the various antigens. They made a series of intradermal injections on the arm, the proximal injection being made with human Frei antigen, two middle injections with egg and mouse brain antigens and two distal injections with the corresponding controls. Reactions were read within forty-eight to ninety-six hours. Of the forty-two patients tested, forty gave a positive, one a doubtful and one a negative reaction to yolk sac antigen; thirty-four gave a positive, five a doubtful, and three a negative reaction to mouse brain antigen; and thirty-eight gave positive, one a doubtful, and three negative reaction to human pus antigen. Thirty-three persons had positive reactions with all three antigens.

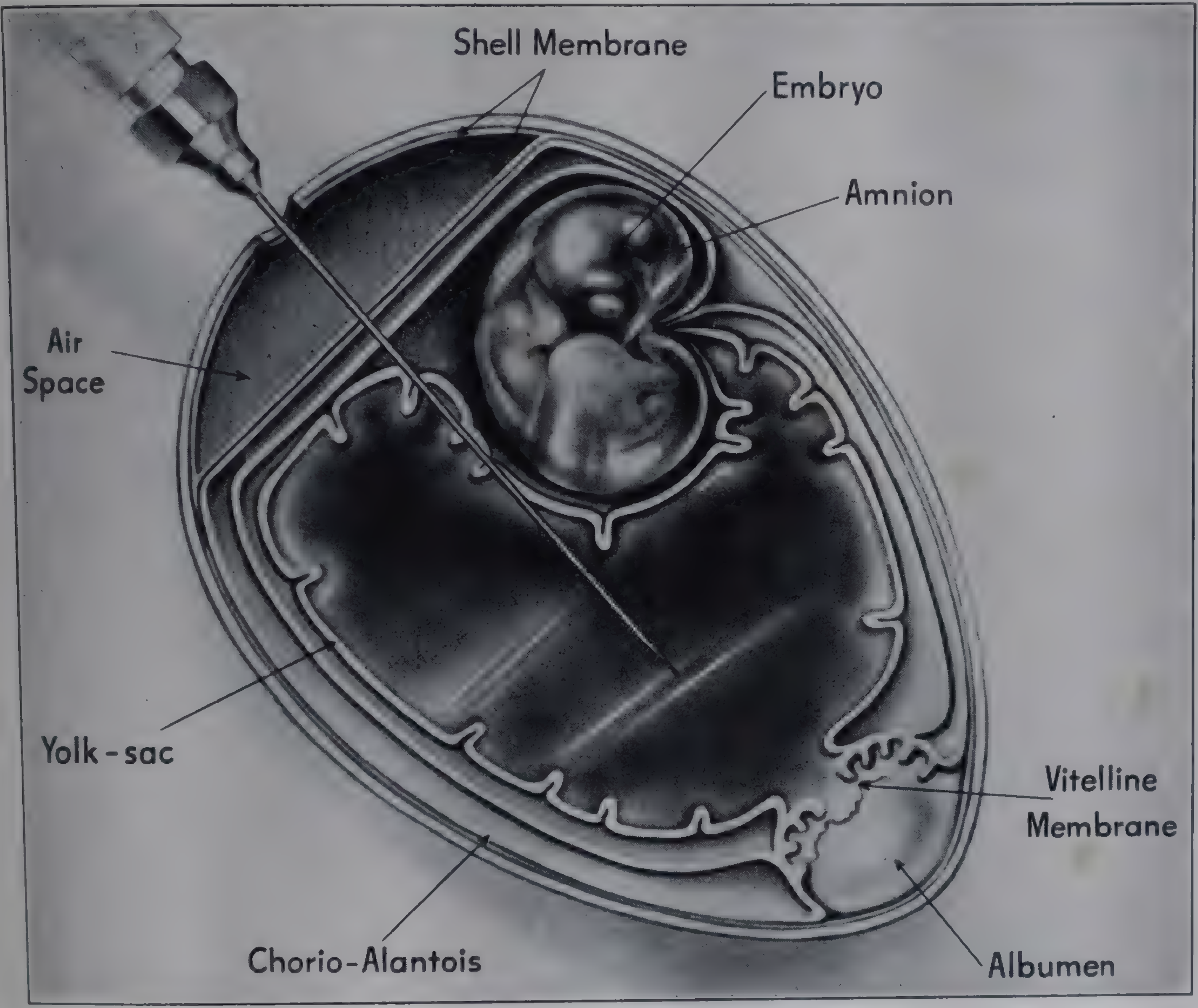

Fig. 468.—Method of injecting the yolk-sac of a developing chick. (Courtesy E. R. Squibb & Sons.)

As these writers point out, any foreign substance when injected intradermally into a human subject will produce an inflammatory response at the site of inoculation and the severity of the reaction will be influenced by any hypersensitivity to the material in the person inoculated. These facts, of course, must be considered in determining the cutaneous response to any Frei antigen. The conclusions of these workers, however, were that the yolk sac antigen is superior to mouse brain antigen for performance of the Frei cutaneous

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*J. A. M. A. **116**: 2663, 1941.

test for lymphogranuloma venereum and that comparatively few nonspecific reactions are produced with the egg control material as compared with the mouse brain control. This antigen is made by Squibb and Lederle, respectively.

Technic of the Test.—Inject 0.1 c.c. of Frei antigen of chick embryo origin intradermally into the flexor surface of the arm. Read reaction within 48 or 72 hours. In order to eliminate false positives due to chick protein sensitivity, a control is supplied by the manufacturer for each test. This consists of normal chick embryo protein prepared in a manner similar to that of the antigen. A positive test is seen by the appearance of a reddish papule 6 mm. or more in diameter, surrounded by a fainter erythematous area of varying size. This papule must be measured with a millimeter scale. The extent and intensity of the erythematous area around the papule is of no diagnostic significance. Strongly positive reactions may have a papule surmounted by a vesicle, or pustule which may break down and form a crust. The reaction will gradually regress, ending as a faintly pigmented area with or without a scar.

The intradermal Frei test is positive only at the peak of the allergic period, when specific antibodies have been formed in the infected organism. This usually becomes positive about 2 weeks after inoculation, sometimes later. It may be negative during the menstrual period and in the not-so-rare cases of florid syphilis if the two diseases coexist.

Interpretation of results should be made from the fourth day. The reaction appears in 24 to 48 hours, reaching a maximum in 3 days, after which it gradually decreases in intensity. A positive reaction consists of a papule, vesicle, or pustule with a diameter larger than 6 mm. It is more intense in inguinal than in rectal cases. The reaction persists for several weeks. Saline control injection should always be made. Nonspecific reactions have a diameter of less than 6 mm. and decrease after three days.

Most of the American workers agree that the chick embryo antigen, Lygranum, is superior to all others for use in the Frei test.

The sole disadvantage of this test is its delayed appearance. Ravaut, Levaditi, and Maisler[1] proposed a hemo-reaction for early diagnosis. Their antigen is injected intravenously, producing a rise of temperature to 102° to 104° F. in 12 to 18 hours which remains for 24 to 48 hours and is accompanied by general malaise, chills, headache, insomnia, weakness, arterial hypotension, and leukopenia. The test has the disadvantage that it can be used only once. It is positive in 97 per cent of the cases.

The complement fixation test may give false positive results, especially in diseases caused by viruses belonging to the same group.

The presence of Miyagawa corpuscles in infected tissues as seen in secretions is considered diagnostic of the disease. These corpuscles can be demonstrated with certain stains: eosin and methylene blue, Macchiavello, Giemsa, Noble.

The blood picture shows leukocytosis, oligocytosis, neutrophilia, lymphopenia, monocytopenia.

The Intradermal Chancroid Test

In connection with the foregoing remarks on lymphogranuloma venereum, it must be remembered that every case of venereal disease requires exhaustive laboratory tests to prove the true diagnosis. It has been found that many cases of chancroid infection have been thought to be lymphogranuloma venereum, but a negative Frei test has caused some doubt concerning this diagnosis. Greenblatt and Sanderson[1] have called attention to a very valuable method of including or excluding chancroid infection in a diagnostic survey of these venereal cases. They have originated an intradermal test for chancroid infection using an antigen made from a saline suspension of the Ducrey bacillus. Their method for growing the Ducrey bacillus is as follows:

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[1] Bull. Soc. franç dermat. et syph. 7: 1262, 1932.

Greenblatt and Sanderson Method of Cultivating Hemophilus Ducreyi

To tubes of beef infusion agar in slants is added 1 c.c. of defibrinated human blood. The blood is inoculated with 0.1 c.c. from an actively growing culture and placed under partial oxygen tension. To obtain this environment, the cotton plug is cut off short and pushed slightly into the tube; the upper portion of the tube is heated, but not too much, in a Bunsen flame, and the tube is sealed by inserting a tightly fitting rubber stopper. The culture is incubated for from two to three days at 37° C. During this period good growth takes place in the fluid blood, and a lesser growth appears on the surface of the slant. After the incubation, the blood, as well as the surface growth, if any, is removed with a sterile pipette and added to approximately 25 c.c. of sterile distilled water in a sterile centrifuge tube of sufficient volume, and the contents are sedimented by high speed centrifugation. The supernatant fluid is removed aseptically and discarded, and the process is repeated. Two such treatments suffice to remove most of the hemoglobin. The sediment of bacilli and red cell debris are suspended in sterile physiologic solution of sodium chloride, 10 c.c. per slant being placed in sterile vials, and heated for thirty minutes at 60° C. A 1:10,000 solution of Merthiolate is used as a preservative. One-tenth cubic centimeter is employed for intracutaneous tests, and the reactions are observed in forty-eight hours.

With this bacillary antigen of Ducrey's bacillus, it is possible to make the diagnosis in venereal cases completely and accurately. It must be remembered that some patients with lymphogranuloma venereum, with a positive Frei test, are also suffering with a chancroid infection, in which case the intradermal chancroid reaction will be present. A positive chancroid test is only presumptive evidence and does not necessarily mean that the lesion under consideration is chancroid. A similar conclusion should be borne in mind in the interpretation of a reactive Wassermann as well as of a positive Frei test in every case of genital lesion unless other confirmatory laboratory evidences are present. A negative intradermal test with bacillary antigen is diagnostic so far as it rules out infection by the bacillus of Ducrey. A positive intradermal chancroid test commits one to the diagnosis of chancroid infection only when the Frei test, the Wassermann test, and microscopic examinations for malignant disease and granuloma inguinale give negative results. This reaction remains positive for life and this fact should be borne in mind in dealing with the above considerations.

GRANULOMA INGUINALE*

Granuloma inguinale is a granulomatous process which usually occupies the inguinal region. It is a mildly contagious, chronic, and progressive autoinoculable disease involving skin and corium and occasionally the lymphatics. Its venereal origin has never been absolutely proved.

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*After Greenblatt in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, St. Louis, 1951, The C. V. Mosby Co.

[1] Arch. Dermat. & Syph. 36: 486, 1937.

The etiological agent is the Donovan body or *Donovania granulomatis*. The disease has been reproduced in human beings by inoculation of aspirated pus from pseudobuboes of granuloma inguinale which contained no other organisms than Donovan bodies. Donovan bodies are strictly tissue parasites of man, but have been successfully cultivated in the yolk sac of developing chick embryos, and less luxuriantly in certain yolk-free media. Inoculation into human volunteers has not been followed by typical reproduction of the disease.

Essential features of the histologic picture are (1) the massiveness of the cellular reaction in which the luxuriant granulation tissue is surcharged with plasma cells; (2) the relative and conspicuous paucity of lymphocytes; (3) the diffuse sprinkling of polymorphonuclear leukocytes, with focal collections in the superficies and papillae; (4) the pronounced marginal epithelial proliferation simulating early epitheliomatous changes; (5) the pathognomonic large mononuclear cells scattered in various numbers throughout the granulation tissue.

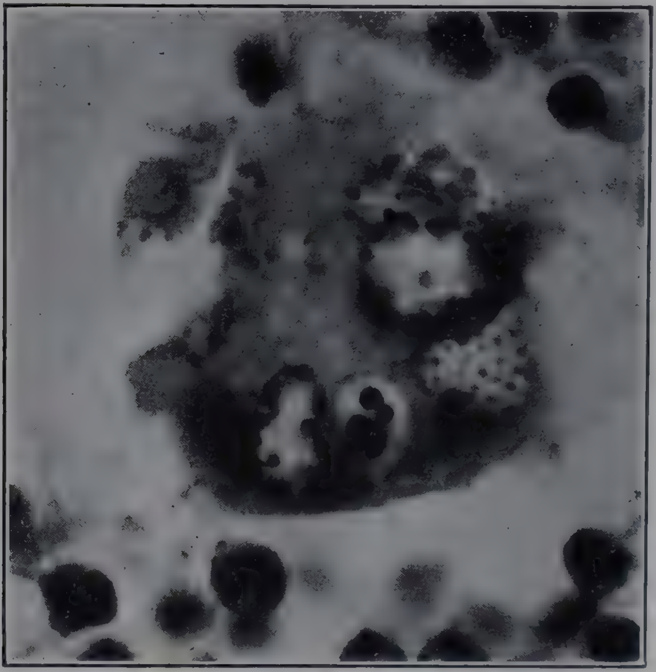

Fig. 409.—Negro male. Granulating lesions of glans penis. Biopsy. Diagnosis: Granuloma inguinale. A pathognomonic cell containing the encysted Donovan bodies. Stained with hematoxylin and eosin (×2,280). (Courtesy Pund, E. R., and Greenblatt, R. B.: Arch. Path., February, 1937.)

The pathognomonic cell is specific for granuloma inguinale. The relatively large size of the cells, the diameter of which varies from 25 to 90 microns, and the many intracytoplasmic cysts filled with deeply stained bodies are its cardinal features according to Greenblatt.[1]

Diagnosis.—

Make spreads by obtaining a minute piece of granulation tissue from the surface of the wound if it appears to be clean, or from the deeper part of the lesion if it appears to be markedly infected.

Remove the exudate covering the infected lesions.

Obtain a small piece of granulation tissue with a sharp instrument such as a small bone curette.

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[1] Pund and Greenblatt: Arch. Path. 23: 244, 1937.

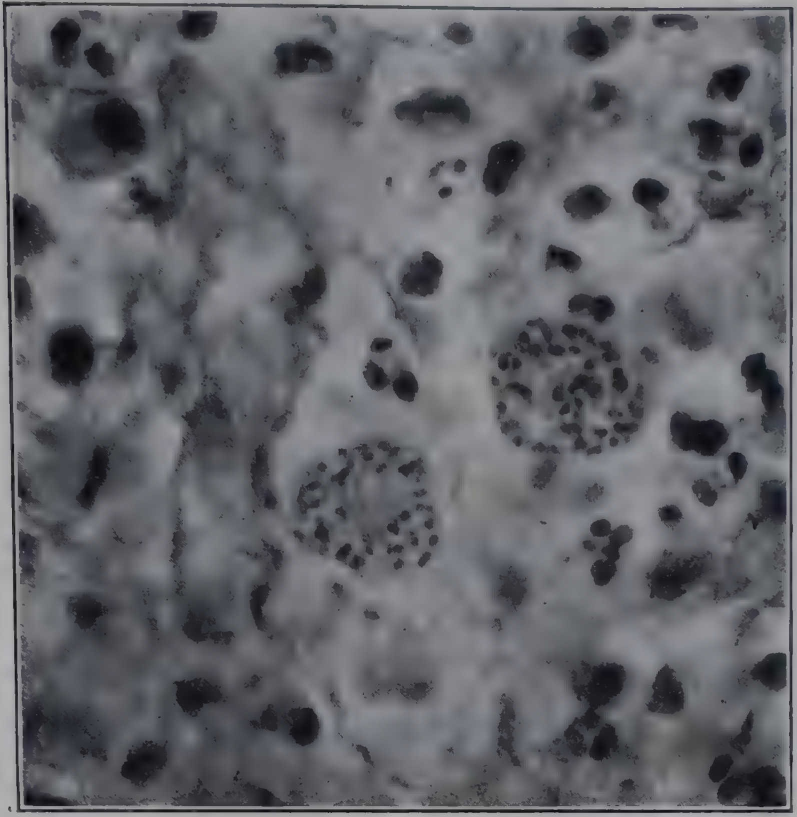

Fig. 410.—Pathognomonic cell of granuloma inguinale containing the encysted Donovan bodies. Stained by Dieterle's silver method. (Courtesy E. R. Pund and R. B. Greenblatt, Personal communication.)

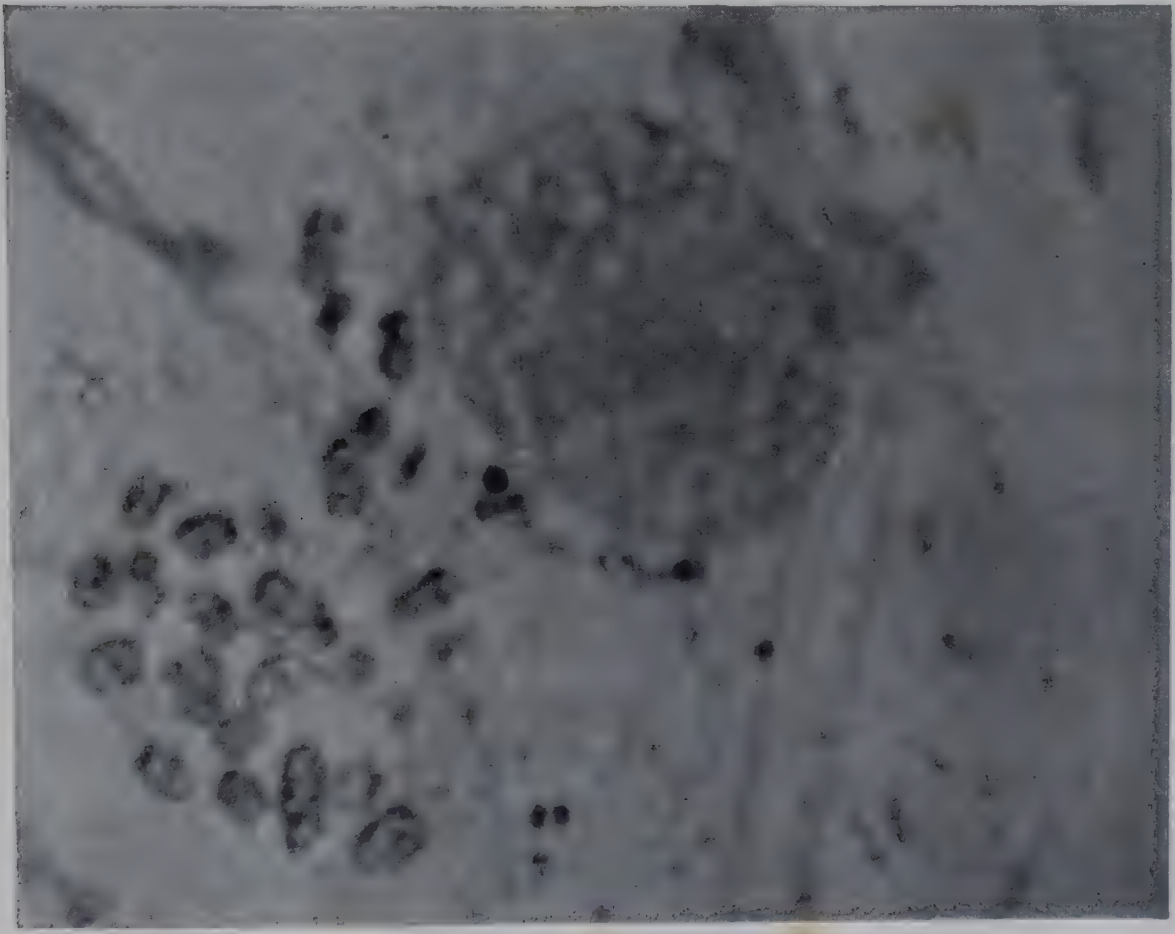

Fig. 411.—Donovan bodies from spreads of lesion (greatly magnified). (From Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Smear the tissue along several glass slides or crush it between two slides and then spread along the slide.

Dry and stain with either Wright or Giemsa stain.* If Giemsa stain is used, the slides must be fixed first in methyl alcohol.

If spreads fail to corroborate the clinical diagnosis, biopsy material may be stained with hematoxylin and eosin or by a silver technic. If the Dieterle method is used,[1] the cell outline, nucleus, and cyst walls stain pale yellow, while the Donovan bodies appear as dark brown or brown rectangular or elongated ovoid forms with intense bipolar staining. Because of their contour and polar characteristics, they resemble a closed safety pin.

YELLOW FEVER†

Yellow fever is an acute infectious disease caused by a specific virus and transmitted by certain species of mosquitoes. It has probably existed in America and Africa since time immemorial. It is believed that the Amazon Valley constitutes a permanent focus of yellow fever, principally maintained in jungle animals. From this enormous endemic region, epidemics originate from time to time and invade the more densely populated areas.

No yellow fever exists in Asia, although the vector, *Aëdes aegypti,* is found there in abundance.

The virus is found in man in the blood, up to the third or fourth day of the disease. It later disappears from the circulation, giving place to neutralizing antibodies. During the period in which it is found in the blood, the virus may infect certain species of mosquitoes which feed thereon.

The virus does not multiply in culture media unless living cells are present.

Yellow fever virus measures approximately 20 millimicrons, being one of the smallest viruses. It remains active for long periods if kept at very low temperatures (–78° C.). Desiccated and kept in sealed glass containers, it remains active several months at 8° C., and almost indefinitely at temperatures below 0° C.

It can cause a disease similar to that in man when injected into rhesus monkeys. It produces a fatal encephalitis when introduced into the central nervous system of white mice. It can be maintained serially by inoculating developing chick embryos a few days old.

Isolation of the Virus.—Take blood from the patient during the first three days of the fever.

Inject 0.03 c.c. intracerebrally into a series of 6 to 12 white mice. If the virus is present, the animals become sick and die usually after a week. It is advisable to make passages with the material from one of the sick mice to another group before trying to make an immunologic identification of the agent.

Neutralizing Test.—

(1) **Identification of the Virus.**—

Prepare suspension in physiologic saline from the brains of mice which have become sick as a result of infection with the virus after several mouse passages.

Make serial dilutions of the suspension, and add a constant quantity of known immune serum to each. Set up a control with normal serum.

Inject each mixture intracerebrally into a group of 6 or 12 mice.

If the immune serum protects the animals in one or more dilutions, the virus is that of yellow fever, provided that mice which receive normal serum and the virus die.

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*See pages 615 and 620.

†Penna, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, St. Louis, 1951, The C. V. Mosby Co.

[1] Arch. Neurol. & Psychiat. **18**: 23, 1927.

(2) **Determination of Specific Antibodies in Serum.**—

Add the unknown serum to a filtered suspension of triturated brains from mice which have become sick after the inoculation of neurotrophic yellow fever virus. Use desiccated material.

Calculate the dilution of the suspension to contain a quantity of virus that can be neutralized by an equal volume of a standard immune serum, and which is sufficient to kill all the animals when mixed with the same volume of normal serum.

Inject 0.03 c.c. of the mixtures of unknown sera and the virus intracerebrally into each of 6 mice.

After a few days, encephalitis can be noted in the animals if the sera do not contain neutralizing antibodies, whereas all mice which receive sera containing such antibodies will remain well.

This test is performed with serum collected during the first days of the illness and again during convalescence. The first sample is usually negative and the second positive.

Viscerotomy.—

In fatal cases, histopathologic examination of the liver should be made, preferably using a "viscerotome."* Specimens are preserved in 10 per cent Formalin.

Complement Fixation.—

Perlowagora and Hughes[1] in 1946 prepared a purified antigen and were able to perform complement fixation tests for yellow fever successfully. Since complement-fixing antibodies disappear from the blood after a relatively short period of time, and neutralizing antibodies persist, it is possible to draw conclusions regarding the probable date of infection by carrying out both tests simultaneously.

Treatment.—

No specific treatment is known against yellow fever. The most practical way of controlling the disease consists in combating the mosquito vector. Vaccination attempts have been unsuccessful.

DENGUE

Dengue is a benign, acute, infectious, noncontagious disease of the tropics, with paroxysmal fever, severe pain in the joints and muscles, multiform and irregular rashes, tendency to relapse, and low mortality. It is also called "break-bone fever," "sun fever," "solar fever," "broken-wing fever," "three-day fever," "bouquet or bucket fever," "eruptive rheumatic fever," "dingee," and "denguis."

It is caused by a virus which passes through most filters and is found in high concentration in the blood during the period of fever. Cultures in the chick embryo have caused lesions on the chorio-allantoic membrane. The virus appears in cultures as globoid bodies 0.1 to 0.3 micron in diameter and arranged singly, in pairs, and in short chains.

The virus retains its virulence in the serum of patients at room temperatures for 1 to 2 months. It resists temperatures as low as −20° C. for at least

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*Penna, loc. cit.

[1] J. Immunol. 55: 103, 1946.

48 hours, and also x-rays and ultraviolet rays. In desiccated blood, it has been known to remain active for at least 285 days. It is destroyed by heat at 50° C. for one-half hour.

Direct transmission of infected blood to normal individuals causes a typical disease, experimental dengue. Mosquitoes become infected when they feed on patients during the first three days of the illness. The virus can be found in the cerebrospinal fluid. Nasopharyngeal exudate and urine have always been negative.

It is transmitted by mosquito vectors.

Immunity is possible, but variable and of short duration.

The red count remains within normal limits, with very slight anisocytosis and poikilocytosis. Leukopenia is constant, the count fluctuating between 1,000 and 4,000 per cu. mm. on the fifth day. There is a relative monocytosis, as high as 14 per cent. There is a left nuclear shift with metamyelocytes and stabs, beginning on the third day. No pathologic forms have been noted. The count returns to normal soon after the beginning of convalescence. An increase in small lymphocytes has been noted by some authors, with a gradual increase of large lymphocytes, and a slight increase of eosinophiles.

Often there are black vomit, hematuria, jaundice, and active delirium.

Degenerative changes occur in the leukocytes.

TRACHOMA BODIES AND OTHER INCLUSION BODIES

Trachoma is a disease of the eye which was well known among the ancient Egyptians, Romans, and Greeks. It has come down to us through the ages and is still a very common disease, not only in modern Egypt but in many places in the United States. It is interesting to recall that, so prevalent was this disease in Egypt, most of the 30,000 grenadiers who swept into Egypt with Napoleon contracted trachoma.

Many attempts have been made to find the etiological agent. It remained for v. Prowazek and Halberstädter, who, during a student journey to Java in 1907, found in the conjunctival sac of trachomatous patients cells which under Giemsa staining showed homogeneous irregular *inclusion bodies* near the nuclei of epithelial cells. These bodies were round or oval, sometimes assuming a mulberry formation. They stained red, often filling up the cytoplasm of the cell in the form of small islands. In smears free bodies were seen. The material in which these bodies were found could be successfully used to inoculate eyes of animals. It is generally considered that these trachoma bodies are protozoan in nature.

Technic.—The technic for demonstration of trachoma bodies is as follows: Clean glassware must be used. After being thoroughly washed, it is finally cleaned in absolute alcohol. The conjunctiva is wiped clear of pus with gauze, not cotton, and then by scraping with a dull knife over the mucosa, enough material is obtained to cover the slide. The purpose is to remove as many pus cells as possible and to obtain epithelial cells from the tissue. The specimen is fixed one to two minutes with methyl alcohol and then stained with dilute Giemsa stain for one to two hours. Permanent specimens are made by covering the slide with cedar oil and then superimposing a cover glass. It is not advisable to use Canada balsam which is likely to decolorize the specimen.

These trachoma bodies may also be demonstrated in sections using Heidenhain iron hematoxylin for staining.

Rice's Method of Demonstration of Inclusion Bodies in Trachomatous Material

This method was suggested by Dr. C. E. Rice,* Medical Officer in Charge, Trachoma Prevention, United States Public Health Service, Rolla, Missouri.

Smears from trachomatous lids are fixed in 95% alcohol. Place several drops of Lugol's solution on slide and drop a thin cover slip over the solution. It can be found under low power and observed under high power. When preparation begins to dry, the cover slip can be flooded off with water, the slide washed slightly in tap water and the smear can again be flooded with Lugol's solution and a cover slip applied. This can be repeated an indefinite number of times.

The inclusions appear as amber-colored bodies, while the cell itself is slightly yellowish. The nucleus can be made out. The amber colored bodies are the so-called von Prowazek-Halberstaedter inclusions found in the epithelial cells in trachoma.

The actively secreting so-called acute trachoma[1] may be divided into three types as follows:

(1) *Chronic trachoma plus superimposed infection.* Contaminating bacteria are found in smear and culture. Inclusions and free bodies rare.

(2) *Subacute trachoma plus superimposed infection.* Secondary bacterial agents found in smear and culture. Inclusions and free bodies often numerous.

(3) *Subacute trachoma, unassociated with pathogenic bacteria.* Inclusions and free bodies numerous.

In chronic trachoma the inclusions and free bodies are found with such difficulty that search for them is not recommended. The fact that large mononuclear cells predominate in expressed follicular materials aids in the differential diagnosis from follicular conjunctivitis in which the lymphocytes predominate.

The Inclusion Bodies.—The cytoplasmic inclusion bodies of trachoma and inclusion conjunctivitis are basophilic and heterogeneous, differing from the inclusions of most other virus diseases which are acidophilic and homogeneous. That this difference is more apparent than real is suggested by the findings of Goodpasture and Woodruff,[2] and Goodpasture, Woodruff, and Buddingh[3] which indicate that such typical virus inclusions as those of vaccinia and fowlpox are composed of masses of elementary virus particles embedded in acidophilic matrices. The elementary bodies of these two diseases, as well as those of psittacosis,[4] closely resemble in size and staining reaction the elementary bodies of trachoma and inclusion conjunctivitis. The discovery that psittacosis virus has a life cycle resembling that of inclusion conjunctivitis virus further emphasizes the essential similarity of the cytoplasmic virus inclusions.

The inclusions of trachoma and inclusion conjunctivitis have in the past been confused with nonspecific bodies such as extruded nuclei,[5] phagocyted nuclear fragments, and phagocyted bacteria.[6] Such confusion is unnecessary if one has had an opportunity to study early cases of inclusion blennorrhea in which the various stages in the development of the inclusions from the initial body form to the elementary body form[7] are easily found. For convenience of reference the inclusions may be spoken of as early (initial body), intermediate (mixed), and late (elementary body).

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*Personal communication.

[1] Thygeson, P.: Arch. Path. 12: 307, 1934.

[2] Am. J. Path. 7: 1, 1931.

[3] Am. J. Path. 8: 271, 1932.

[4] Bedson, S. P., and Bland, J. O. W.: Brit. J. Exper. Path. 8: 461, 1932.

[5] Gifford, S. R., and Lazar, N. K.: Arch. Ophth. 4: 468, 1930.

[6] Williams, A.: J. Infect. Dis. 14: 261, 1914.

Free elementary and initial bodies are frequently useful in diagnosis, particularly in acute trachoma and in inclusion blennorrhea in which they often appear in large numbers. The initial bodies stain blue and bipolarly with Giemsa stain and are cocco-bacillary in shape. The elementary bodies are small granules about 0.25 μ in diameter, which stain reddish blue with Giemsa stain. They may be identified from their uniform size, frequent diplococcal form, and the fact that they are alcohol-fast, while nonspecific granules, such as neutrophilic granules, decolorize readily.

Inclusion Blennorrhea

This affection is also, like trachoma, very common. It is an intrapartum affection of newborn children contracted from mothers infected with this disease. It has been well studied by Lindner. Five or six days after birth, clinical symptoms appear in the form of redness and swelling in the region of the conjunctiva with a purulent secretion.

The bodies resemble trachoma bodies in every particular. Thygeson, of Columbia University, has described the elementary body as follows: it is similar in size and staining reaction to elementary bodies of such virus diseases as vaccinia, fowl-pox, and psittacosis. They are minute, sharply defined granules of uniform size, often in pairs, staining reddish blue with Giemsa stain. The average diameter is about 0.25 micron. The so-called initial body is similar in size and staining characteristics to the trachoma body and to the large form of psittacosis virus. It is cocco-bacillary in shape, staining blue and bipolarly with Giemsa stain.

Essential characteristics of elementary and initial bodies: gram-negative; stain poorly with aniline dyes; alcohol-fast. Not cultivable on artificial media.

The initial body is the early phase of the virus, the elementary body the late phase. The elementary body is the infective stage. Its life cycle is as follows: (1) Penetrates epithelial cell. (2) Grows to initial body size. (3) Multiplies, the individual elements gradually becoming smaller and smaller until (4) Elementary body stage is reached. (5) Cell ruptures and liberates elementary bodies.

Multiplication of Virus During Incubation Period.—Three complete life cycles from elementary body to elementary body were demonstrated during six-day incubation period of an experimental infection. The life cycle, therefore, is probably 48 hours.

Filtrability of Virus.—The virus filters readily only during the acute stage when free elementary bodies are abundant. In chronic stages elementary bodies are in insufficient numbers to saturate absorptive properties of the filter and thus are all retained in the filter.

Inclusion blennorrhea is a birth canal infection. This has been proved by the demonstration of inclusion bodies and free virus in vaginal scrapings from mothers of infected infants and also by infection of monkey conjunctiva with vaginal secretions.

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[7] Thygeson, P.: Etiology of Inclusion Blenorrhea, the Transactions of the Association for Research in Ophthalmology, 1934.

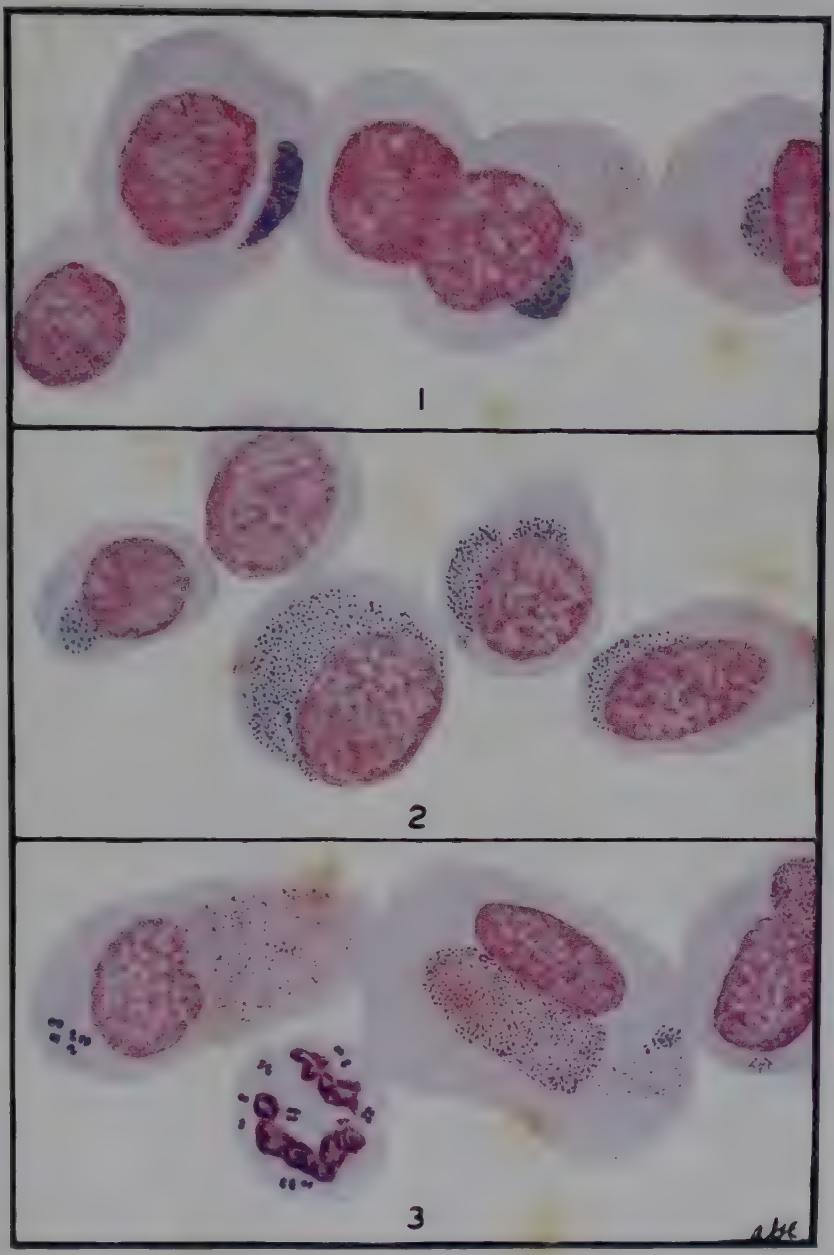

Plate XLVI.—Giemsa-Stained Preparations of Various Types of Inclusions.

(1) Trachoma; (2) Inclusion Blennorrhea of the Newborn; (3) von Prowazek-Halberstädter Bodies and Gonococci.

Technic.—Scrape epithelia from conjunctiva of baby by anesthetizing with drop of cocaine. Fix in methyl alcohol for ten minutes. Stain overnight in the incubator with Giemsa stain at 37° C. Dilute Giemsa stain 1 drop to each c.c. of neutral distilled water before using. Decolorize in 95% alcohol in two solutions for a total of five seconds. Dry in the air and examine.

Inclusion Bodies of Swimming-Pool Conjunctivitis

Swimming-pool conjunctivitis was described by Fehr in 1900.[1] It has frequently been described since then and has occurred in epidemics from bathing in swimming pools. The diagnosis clinically rests upon an acute beginning and the remarkable discrepancy between the slight subjective symptoms and the marked objective changes—such as marked swelling of the conjunctiva, overfilling of the follicles, etc. Since the advent of swimming pools into our community life, it is well to be on the lookout for this disease. The virus is evidently a body very similar to the trachoma and the inclusion blennorrhea bodies described by Lindner. This ''swimming pool'' inclusion body was first described in 1913 by Huntemuller and Pederstein.[2] They called attention to the similarity of this body to the Halberstädter body. Thygeson is of the opinion that the term ''inclusion conjunctivitis'' should include the two nonbacterial conjunctival diseases, inclusion blennorrhea and swimming-pool conjunctivitis, which are caused by a single virus. The name ''swimming-pool conjunctivitis'' should be discarded in favor of ''adult inclusion conjunctivitis'' since the swimming pool no longer plays a major part in the spread of the disease. The majority of cases now seen are the result of direct transfer from inclusion disease of the genitourinary tract.

The demonstration of the inclusion bodies of swimming-pool conjunctivitis is carried out just as described above for inclusion blennorrhea.

This disease usually appears as a follicular conjunctivitis, subacute at onset. Occasionally, however, it is seen as a papillary conjunctivitis in which follicles are either absent or masked by the papillary hypertrophy. The latter type is the more severe. Inclusions are often abundant in the early stages although never in such numbers as in the disease of the newborn. In the former type the inclusions are few in number and are commonly found only during the first few weeks of the disease. Free elementary and initial bodies are sometimes present even when inclusions are not demonstrable.

It is this type of follicular conjunctivitis which is most often confused with trachoma. The examination of expressed follicular contents after staining with Giemsa is a useful diagnostic procedure. In trachoma the large mononuclear cells predominate, while in all forms of follicular conjunctivitis the small mononuclear cells are the most numerous. In the trachoma follicle the central portion, made up of large mononuclear cells, has undergone gelatinous degeneration and expresses easily. The follicles in follicular conjunctivitis, on the other hand, are hard and have to be torn out in toto. The small mononuclear cells making up the peripheral portions of the follicles then appear in greatest number.

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[1] Berlin klin. Wchnschr. No. 1, p. 10, 1900.
[2] Deutsche med. Wchnschr., p. 63, 1913.

Results Obtainable with Combined Film and Culture Technic.—Some indication of the results to be expected from the technic described is given by the following figures derived from the study of 314 consecutive cases of conjunctivitis at the ophthalmologic clinic of the University Hospital, Iowa City:

	Etiological Agent Determined in
Acute conjunctivitis	91% of cases
Subacute conjunctivitis	85% of cases
Chronic conjunctivitis	46% of cases

SUMMARY OF THE LABORATORY DIAGNOSIS OF VIRAL DISEASES

The procedures followed in the laboratory diagnosis of diseases caused by viruses are analogous to those used in bacterial infections. Conclusive evidence consists in the demonstration and identification of the virus. This may be accomplished in some of the virus diseases (psittacosis, yellow fever, influenza, and rabies) by using susceptible laboratory animals or chick embryos instead of the ordinary culture media used for the isolation of bacteria. Unfortunately, many factors complicate the use of animals in virus study: (1) costliness of animals often prohibits their use (monkeys in poliomyelitis); (2) in some cases animals must be strictly isolated to prevent contact infections (Russian spring-summer encephalitis); and (3) latent viruses of ordinary laboratory animals frequently confuse diagnosis.

Once the virus is recovered, identification is accomplished by determination of the animals for which it is pathogenic, routes by which it can be injected, tissues it infects, pathologic lesions produced, and through the use of the classical immunologic reactions, viz., neutralization, complement fixation, agglutination or precipitation reactions. The neutralization or so-called "protection test" was the first immunologic phenomenon to be used in connection with virus diseases (Babes and Cerchez,[1] Tizzone and Schwartz[2]). The complement fixation test came into use in 1906 when Jobling[3] showed that the serum from individuals convalescent from vaccinal infection would fix complement after being mixed with vaccine virus. A few years later Paschen[4] showed that elementary bodies of vaccinia and smallpox were specifically agglutinated by serum from individuals convalescing from these infections.

Microscopic study of virus-containing tissues is of value in identification and in establishing the relationship of the virus to the human disease under investigation. Certain viruses producing apparently identical lesions, however, may be distinguished only by their species specificity or their immunologic reactions. Inclusion bodies are not in all cases distinct enough from the morphologic standpoint to serve as characteristic indicators of the presence of virus. Furthermore, these bodies have not been demonstrated in a number of virus diseases; they have been found often in pathologic material in which the presence of a virus could only be suspected; and they have been produced in tissues in which the presence of a virus could be excluded with reasonable certainty (Olitsky and Harford[5]). When a virus can be demonstrated by passage as indicated above, the presence of inclusion bodies may serve as corroborative evidence.

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[1] Ann. Inst. Pasteur 5: 625, 1891.

[2] Ann. de Micrographie 4: 169, 1891.

[3] J. Exper. Med. 8: 707, 1906.

[4] Deutsch. Med. Wchnschr. 39: 2132, 1913.

Neutralization Tests.—Neutralization tests are generally used for detecting the presence of virus-neutralizing antibodies for diagnostic or epidemiologic purposes. The serum to be tested is mixed with the virus in suitable proportions and then inoculated into susceptible animals. In some of these tests the amount of virus used is kept constant and different dilutions of serum are added, while in others the amount of serum is kept constant and the virus diluted. In general, the basis upon which such neutralization tests are interpreted depends upon the susceptibility of the animal used. Usually, when the virus is highly pathogenic, the results are interpreted according to the proportion of animals which survive. With some viruses it has been possible to utilize skin reactions for titrating the antibody content of immune serum, thus permitting one to carry out several experiments simultaneously on a given animal. This procedure was used by Gordon[6] in his studies with vaccinia virus and by Van Rooyen and Rhodes[7] with rabbit myxoma and Shope's fibroma viruses. More recently the chorio-allantoic membrane of the developing chick embryo has been used for virus neutralization tests by Burnet[8] and by Blattner and Cooke.[9]

Olitsky and Harford[10] have shown that demonstration of protective antibodies is more effective when serum-virus mixtures are given by some routes of inoculation than by others. This fact must be considered in carrying out practical diagnostic tests.

Frequently a specimen of serum can be obtained only during convalescence. In such cases, the validity of the diagnosis depends on the frequency with which sera from apparently normal individuals in a given community give positive tests. In attempting a diagnosis of St. Louis encephalitis or lymphocytic choriomeningitis by single specimens of serum taken during convalescence it must be remembered that the sera of many normal persons of the same community also give positive results (Wooley and associates[11]). During epidemic periods, because of subclinical exposure, a large percentage of normal persons may develop antibodies against the virus responsible for the outbreak. Accordingly, based on the study of serum specimens from the general population and from cases of influenza occurring during the 1940-1941 outbreak in St. Louis, the level of complement fixing antibodies near a titer of 1 to 16 in terms of the original dilution of the patient's serum was found to be significant of epidemic influenza (Sulkin and associates[12]). In certain virus diseases specific antibodies develop quite rapidly (St. Louis encephalitis and equine encephalomyelitis), while in others, antibodies may not become apparent for several weeks or even months. The technic of neutralization tests is given on page 1752.

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[5] Am. J. Path. **13**: 729, 1937.

[6] Med. Res. Council, Special Reports, Series No. 98, H. M. Stationery Office, London, 1925.

[7] Zbl. Bakt., Abt. 1, orig. **142**: 119, 1938.

[8] Med. Res. Council Spec. Reports, Series No. 220, H. M. Stationery Office, London, 1936.

[9] J. Infect. Dis. **70**: 226, 1942.

[10] J. Exper. Med. **68**: 173, 1938.

[11] Ibid. **54**: 938, 1939.

[12] Loc. cit.

In Vitro Tests.—The early literature contained many conflicting reports concerning the application of complement fixation tests to the study of virus diseases. In subsequent years, however, various improvements have been introduced which have increased the practicability and resulted in the more extensive use of this test. Merrill[13] has indicated that theoretically in vitro immunologic reactions should be demonstrable, provided a sufficient quantity of virus is contained in the tissue used as antigen. However, if the concentration of virus in the tissues is low, a delicately balanced system must be used as suggested by Bedson.[14] Sufficient time must be allowed for the union of antigen and antibody. Accordingly, Bedson and Bland[15] permitted complement fixation to occur over a period of hours at low temperature, employing the procedure previously used for increasing the delicacy of the Wassermann test.

COMPLEMENT FIXATION TESTS FOR RICKETTSIAL AND VIRAL DISEASES

Complement fixing antibodies begin to appear in the blood of the patients usually in the middle of the second week of the illness. Titers of 1:10 or higher are indicative. The test should be repeated at about 10-day intervals, whether the reaction has been negative or not. Neurotropic viruses will seldom give titers higher than 1:30.

Antigen.—

The principal difficulty in applying the complement fixation technic to viral and rickettsial diseases has been the preparation of suitable antigens. DeBoer and Cox,[16] and later Van der Scheer, Bohnel, and Cox,[17] have developed a method of preparing antigens in which nonspecific reactions are reduced and by which the rickettsial antigens may be purified and concentrated. Further studies led to commercial production of usable antigens, of which those of Lederle Laboratories are recommended.[18] The following list gives information concerning these antigens and the tissue utilized in preparing them.

Antigen	Tissue Source
Equine encephalomyelitis (Eastern) (EEE)	Whole chick embryo
Equine encephalomyelitis (Western) (WEE)	Whole chick embryo
Japanese B encephalitis (Jap B)	Mouse brain
St. Louis encephalitis (SL)	Mouse brain
Lymphocytic choriomeningitis (LCM)	Guinea pig lung
Mumps	Chick embryo—chorio-allantoic fluid
Influenza (all available strains)	Chick embryo—chorio-allantoic fluid
Psittacosis (PSI)	Chick embryo—chorio-allantoic fluid and membrane
Q fever (all available strains)	Purified rickettsial suspension
Rickettsialpox (RP)	Chick embryo—yolk sac
Rocky Mountain spotted fever (RMSF)	Chick embryo—yolk sac
Typhus (both strains)	Chick embryo—yolk sac

Sheep Cells.—

Collect sheep blood in a bottle containing a few glass beads and shake thoroughly. Filter through several layers of sterile gauze and store in a refrigerator.

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[13] J. Immunol. **30**: 169, 1936.

[14] Brit. J. Exper. Path. **9**: 235, 1928.

[15] Brit. J. Exper. Path. **10**: 393, 1929.

[16] J. Immunol. **55**: 193, 1947.

[17] J. Immunol **56**: 365, 1947.

[18] Antigens for these tests are obtainable from the Lederle Laboratories Division of the American Cyanamid Company, 30 Rockefeller Plaza, New York 20, N. Y., and are recommended for routine use.

This suspension is suitable for use for 5 to 6 days. Sheep cells defibrinated and stored in this manner have been found more satisfactory for use with the present antigens than citrated cells or blood stored in various preservatives.

Remove adequate portions as required and wash in the usual manner (page 2048) with saline, until the supernatant fluid is colorless.

Suspend the cells in the proportion of 2 c.c. of packed cells to a total of 100 c.c., using 0.85% saline as the diluent.

The washed cells, if stored in a refrigerator at 6° to 8° C., may be used for two successive days.

Titration of Hemolysin.—

Prepare serial dilutions of antisheep hemolysin, as in the Kolmer test (page 2051).

Prepare 1:30 dilution of complement as in the Kolmer test (pages 2049, 2051).

Carry out the titration as follows:

TUBE NUMBER	HEMOLYSIN		1:30 COMPLEMENT C.C.	SALINE C.C.	2% SHEEP CELLS C.C.
	DILUTION	C.C.			
1	1:1,000	0.25	0.25	0.75	0.25
2	1:2,000	0.25	0.25	0.75	0.25
3	1:3,000	0.25	0.25	0.75	0.25
4	1:4,000	0.25	0.25	0.75	0.25
5	1:5,000	0.25	0.25	0.75	0.25
6	1:6,000	0.25	0.25	0.75	0.25
7	1:8,000	0.25	0.25	0.75	0.25
8	1:10,000	0.25	0.25	0.75	0.25
9	1:12,000	0.25	0.25	0.75	0.25
10	1:16,000	0.25	0.25	0.75	0.25

Incubate at 37° C. in a water bath for 1 hour.

Read. The highest dilution of hemolysin which causes complete hemolysis at the end of an hour is *one* unit. Use *two units* in all subsequent titrations and in the test.

Example: If tube 4 shows complete hemolysis and tube 5 shows only partial or slight hemolysis, one unit of hemolysin is 0.25 c.c. of a 1:4,000 dilution. Two units would be 0.25 c.c. of a 1:2,000 dilution. It is not advisable to use the sheep cells if the titer is lower than 0.25 c.c. of a 1:1,000 dilution; new cells must be prepared.

Preparation and Titration of Complement.—

Dilute the complement 1:30 with saline, as for the Kolmer test, pages 2049, 2051.

TUBE	1	2	3	4	5	6	7	8	9	10
Complement (1:30) c.c.	0.100	0.125	0.150	0.175	0.200	0.225	0.250	0.275	0.300	0.325
Saline c.c.	0.650	0.625	0.600	0.575	0.550	0.525	0.500	0.475	0.450	0.425
Antigen 2 units c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Incubate in a water bath at 37° C. for 1 hour.										
Hemolysin 2 units c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
2% sheep cells c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Incubate in a water bath at 37° C. for 1 hour.										

Read. The smallest quantity of 1:30 dilution of complement which shows complete hemolysis is *one exact unit.* Use *two exact units,* contained in 0.5 c.c., for all antigen titrations and for the test.

If the complement is prepared in the laboratory from fresh guinea pig blood, the overnight-icebox incubation should be used instead of the first incubation in a water bath, and any serum rejected if it shows less complement activity than average in the presence of 2 units of a given antigen.

Use the following table to determine the required complement dilution so that 0.5 c.c. contains exactly 2 units.

ONE EXACT UNIT OF COMPLEMENT	TWO EXACT UNITS OF COMPLEMENT
0.100 c.c. of 1:30 dilution	0.5 c.c. of 1:75 dilution
0.125 c.c. of 1:30 dilution	0.5 c.c. of 1:60 dilution
0.150 c.c. of 1:30 dilution	0.5 c.c. of 1.50 dilution
0.175 c.c. of 1:30 dilution	0.5 c.c. of 1:42.8 dilution
0.200 c.c. of 1:30 dilution	0.5 c.c. of 1:37.5 dilution
0.225 c.c. of 1:30 dilution	0.5 c.c. of 1:33.3 dilution
0.250 c.c. of 1:30 dilution	0.5 c.c. of 1:30 dilution
0.275 c.c. of 1:30 dilution	0.5 c.c. of 1:27.5 dilution
0.300 c.c. of 1:30 dilution	0.5 c.c. of 1:25 dilution
0.325 c.c. of 1:30 dilution	0.5 c.c. of 1:15 dilution

The Diagnostic Complement Fixation Test.—

To make serial dilutions of the patient's serum, make a 1:4 dilution in saline by adding 0.3 c.c. of the freshly inactivated serum to 0.9 c.c. of saline.

Rack up 6 tubes for the test, 3 for the serum controls, and 3 for other controls.

Place 0.25 c.c. of saline in tubes 2 through 6, and in tubes 8 and 9.

Place 0.25 c.c. of the 1:4 dilution of patient's serum in tube 1 and in tube 2 of the test (first six tubes) and in tubes 7 and 8 of the controls.

Mix the contents of tube 2 and transfer 0.25 c.c. to tube 3, mix and transfer 0.25 c.c. to tube 4, and continue through tube 6. Discard the extra 0.25 c.c. of diluted serum.

Mix the contents of tube 7, transfer 0.25 c.c. to tube 8, mix, and transfer 0.25 c.c. to tube 9. Mix and discard 0.25 c.c. of diluted serum.

Tubes 1 through 9 now each contain 0.25 c.c. of various dilutions of serum as follows:

Tube	1	2	3	4	5	6	7	8	9
Dilutions	1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16

Prepare the diagnostic complement fixation test indicated in Table 167, adding the reagents in the order listed.

TABLE 167.—DIAGNOSTIC COMPLEMENT FIXATION TEST FOR RICKETTSIAL AND VIRAL DISEASES

	TEST						SERUM CONTROLS			OTHER CONTROLS		
	1	2	3	4	5	6	7	8	9	10	11	12
Serum dilutions	1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16	a	b	c
c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	–	–	–
Antigen 2 units c.c.	0.25	0.25	0.25	0.25	0.25	0.25	–	–	–	0.25	–	–
Complement 2 units c.c.	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Saline c.c.	–	–	–	–	–	–	0.25	0.25	0.25	0.25	0.50	0.75
Incubate overnight at +6° to +8° C.												
Hemolysin 2 units c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	–
2% Sheep cells c.c.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Incubate at 37° C. for 15 minutes in a water bath												

Read.

One set of serum controls is run for each serum sample, and one set, in duplicate, of other controls for all sera.

Tube *a* is the antigen control, and should show complete hemolysis.

Tube *b* is the complement control and should show complete hemolysis.

Tube *c* is the sheep cells control and should show complete absence of hemolysis.

The highest dilution of the patient's serum exhibiting 3 or 4 plus reaction—between 75 and 100% inhibition of hemolysis—represents the serum titer.

Whereas various nonspecific and zone reactions may seemingly occur if the tests are read as soon as the antigen and serum control tubes show complete hemolysis, "true" reactions will persist longer.

Allow the tubes to stand at room temperature for 1 hour following the first reading, and read again. "True" reactions should persist for this additional length of time.

A known positive serum must be included as a control for each antigen utilized in the diagnostic test. It is advisable to use the same positive serum each time a particular antigen is used. Thus, a definite rise or fall in titer exhibited by the control serum will be indicative of a faulty test run.

If a question arises as to whether a positive reaction is due to the organ content of an antigen rather than to the etiological agent, a control for specificity must be included. Refer to the table, page 1742, showing the tissue source of each antigen, and select an antigen which will serve as a control for another. For example, equine encephalomyelitis, Eastern, may serve as a control for equine encephalomyelitis, Western; mumps, psittacosis, and influenza can control each other; rickettsialpox, or Rocky Mountain spotted fever, can control either of the typhus strains. Japanese B encephalitis and St. Louis encephalitis must be compared with normal mouse brain antigen.

The Use of the Chick Embryo as an Aid in Diagnosis.—The fertile egg is now widely used and has been found to be applicable to the study of a variety of virus problems. One of the first publications on the use of chick embryos for studying bacterial infections was that of Levaditi[19] in 1906. According to this investigator, however, chick embryos were used the previous year by Borrel. Subsequently they were used by a number of investigators including Rous and Murphy,[20] Juan and Staub,[21] Gay and Thompson,[22] and others, but the method was generally introduced into bacteriology by Goodpasture and his associates[23] in their study of fowl pox virus.

The chick embryo is now widely used for the isolation, cultivation, and titration of viruses; for titration of antiviral sera; as a source of antigen for immunologic procedures; for the preparation of vaccines for human use; and for studying the development of lesions. The reader is referred to the articles by Goodpasture and his associates,[23] Burnet,[24] Stevenson and Butler,[25] and Bengtson and Dyer.[26] Convenient histologic methods of examining infected chorio-allantoic membranes have been described by Goodpasture and his coworkers,[27] Himmelweit,[28] and more recently Burnet and Faris.[29] Cooke and Blattner[30] have described a method of staining virus foci on chick membranes. The staining procedure, using trypan blue, facilitates the recognition and enumeration of lesions on the developing chick membrane. See pp. 1473 ff.

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[19] Ann. Inst. Pasteur **20**: 924, 1906.

[20] J. A. M. A. **56**: 741, 1911.

[21] Ann. Inst. Pasteur **34**: 343, 1920.

[22] Proc. Soc. Exper. Biol. & Med. **26**: 556, 1929.

[23] Ibid **21**: 319, 1935.
Ibid. **28**: 111, 1938.

[24] J. Path. & Bact. **37**: 107, 1933.

[25] Rep. Pub. Health Med. Subj., London, No. 87, 1939.

[26] Pub. Health Rep. **50**: 1489, 1935.

[27] Loc. cit.

[28] Brit. J. Exper. Path. **19**: 108, 1938.

[29] J. Bact. **44**: 241, 1942.

[30] Am. J. Path. **18**: 163, 1942.

Egg Culture Methods.—Egg culture methods are described in detail on pages 1473 ff. Method of injecting into the yolk sac is shown on page 1729.

Preservation of Viruses.—It has long been known that viruses which are unstable and rapidly become inactivated at room temperatures retain their infectivity for longer periods at lower temperatures. The most widely used method of preservation is 50 per cent glycerin.* This method is simple, but does not maintain the infectivity of some viruses over long periods.

A technic for the preservation of various bacteria, filtrable viruses, and other biologic preparations by desiccation from the frozen state was developed through the studies of Shackel,[31] Swift,[32] Sawyer and associates,[33] Elser and associates,[34] and more recently by Flosdorf and Mudd.[35] The latter investigators have described procedures and apparatus for preservation of viruses and other biological materials by drying while in the frozen state. This system, known as the "lyophile process," has been used effectively by Wooley.[36] Flosdorf and Mudd[35] later described the so-called "cryochem process," a further refinement of the original method. This procedure has since been used for the preservation of numerous types of infectious materials, and has been found to be highly effective for most viruses.

Carbon dioxide in solid form has been used to maintain virus-containing materials at very low temperatures with conspicuous success. The specimen tubes are surrounded by a mixture of alcohol and carbon dioxide snow. The tubes containing the material are rotated, so that the contents freeze uniformly. Then a vacuum is attached and the tubes sealed. They may be stored in a freezer without vacuum desiccation.

Common Errors in Laboratory Procedure.—The diagnosis of virus diseases is subject to numerous difficulties, both in the handling of specimens and in procedure of the various tests. These pitfalls may arise from complications inherent in the virus itself or in the nature of the test being used. The worker must therefore have a clear understanding of the mechanism of any test undertaken. In the following discussion, some of the more common errors in laboratory diagnosis will be pointed out.

One frequent error occurs when a latent virus appears during animal passage and is mistaken for that causing the human disease. Several spontaneous infections in apparently healthy mice from various highly inbred laboratory stocks have complicated experimental study of viruses.

Authorities differ as to the amount of incubation of serum-virus mixtures necessary in the performance of neutralization tests. Although some workers advocate definite incubation periods, good results have been obtained where only sufficient time to make necessary manipulations was allowed. Incubation may cause some inactivation of the virus so that it is important that control animals inoculated with normal serum-virus mixtures be carefully observed. It is desirable to inoculate the immune serum-virus mixtures first so that any protection observed cannot be attributed to standing during inoculation of control animals.

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*It is not necessary to use Schering-Kahlbaum brand of glycerin, but it is desirable to use a pure good grade.

[31] Am. J. Phys. **24**: 325, 1909.

[32] J. Exper. Med. **33**: 69, 1921.

[33] J. Exper. Med. **50**: 1, 1929.

[34] J. Immunol. **28**: 433, 1935.

[35] J. Immunol **29**: 389, 1935.
Ibid. **34**: 469, 1938.

[36] Pub. Health Rep. **54**: 1077, 1939.

The diluent used for suspending the virus-containing tissues is an important factor and is almost always stated. In general, 1 to 10 per cent solutions of normal horse or rabbit serum in saline are satisfactory, but it must be made certain that the serum does not contain specific antibodies against the virus used. Infusion broth is also valuable, but in some instances has been found to be inferior to serum. Beef infusion broth containing 20 per cent horse serum has been found satisfactory in studies with influenza virus.

Although specific complement fixing antibodies have been demonstrated in many virus diseases, these reactions are so delicate that the execution of the tests, their adequate control, and interpretation of results demand utmost care and attention. One of the chief obstacles remains that of nonspecific complement fixation caused by tissue proteins from which viruses are inseparable. For example, the appearance of certain immunologic effects, such as the Forssman phenomenon, may interfere with the interpretation of the results of serologic reactions. The animal species from which antigen is derived and animal species from which antibody is obtained must be considered. Fairbrother and Hoyle[37] found from their control experiments that the complement fixation which occurred between anti-influenzal ferret serum and influenzal mouse lung was nonspecific in character as the same effect was obtained with normal mouse lung as antigen. Therefore, suitable antigen and serum controls must be included before the results of these tests can be interpreted. In addition, unless the same pooled complement is used throughout a series of tests, comparisons cannot be made, as the deviability of different specimens of complement is likely to vary considerably.[38] Refer to page 1745.

Many workers in the fields of biology and medicine believe that if results need statistical evaluation they are of such inconclusive nature that they should not be considered valid. On the other hand, we are of the opinion that results which look significant may well be given the test of statistical analysis since occasionally they may not prove to be as conclusive as they appear. Mainland[39] discusses the use of statistical methods in such situations. Furthermore, in the primary isolation of a virus, it is desirable to use a large number of animals. This obviously increases the possibility of obtaining positive results, and if all animals show signs of infection, activation of a latent virus is less likely to have occurred. Again, in making serum protection tests, the use of a large enough number of animals makes the conclusions less open to criticism. Schaeffer and Muckenfuss[40] have shown how the difficulty in using large numbers of monkeys in experimental poliomyelitis has made results in many cases invalid.

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[37] J. Path. & Bact. 44: 213, 1937.

[38] J. Exper. Med. 13: 45, 1911.

[39] The Treatment of Clinical Laboratory Data. An Introduction to Statistical Ideas and Methods for Medical and Dental Workers, Oliver and Boyd, 1938.

[40] Loc. cit.

TABLE 168.—CLASSIFICATION OF HUMAN VIRUS DISEASES (AFTER SULKIN)[1]

Based on their possible modes of transmission, entry into the body, and methods of excretion

MODES OF TRANSMISSION TO NEW HOST†

AIR BORNE	FOMITES	DIRECT CONTACT	INSECT VECTORS	ANIMAL RESERVOIRS	LABORATORY INFECTIONS
*Influenza	*Variola	*Rabies	*Yellow Fever	*Rabies	*Psittacosis
*Common Cold	*Psittacosis	*Lymphogranuloma venereum	*Dengue	*Psittacosis	*Yellow Fever
*Measles	*Molluscum contagiosum	*Molluscum contagiosum	*Poliomyelitis	*B Virus	*Lymphogranuloma venereum
*Psittacosis	Herpes	Warts	*Rift Valley Fever	*Equine encephalomyelitis	*Lymphocytic choriomeningitis
*Mumps	Varicella	Herpes genitalis	*Sand-Fly fever	*Lymphocytic choriomeningitis	*Equine encephalomyelitis
Variola	Rabies	Inclusion blennorrhea	*Forest-Spring encephalitis‡	*Foot & mouth disease	*B Virus
Varicella	Foot & mouth disease	Foot & mouth disease	Equine encephalomyelitis	*Louping ill	*Influenza
Herpes	Trachoma††	Lymphocytic choriomeningitis	St. Louis encephalitis	St. Louis encephalitis	*Louping ill
Louping ill	Inclusion blennorrhea	Australian X disease	Japanese B encephalitis	Influenza	*Rift Valley fever
Primary atypical pneumonia**			Australian X disease		Primary atypical pneumonia**
Poliomyelitis			Lymphocytic choriomeningitis		Poliomyelitis
Lymphocytic choriomeningitis			Louping ill‡		Newcastle disease
St. Louis encephalitis			Molluscum contagiosum		
Japanese B encephalitis			Psittacosis		
Australian X disease			Herpes		
Foot & mouth disease			Trachoma		
Coxsackie			Rabies		

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*Diseases in which epidemiological data appear to be fully established.

**Refers to the so-called "virus pneumonias" although a virus has usually not been recovered.

†Certain virus diseases are omitted from this classification because the mode of transmission is unknown.

‡There are some indications that Russian Forest-Spring Encephalitis is the same as Louping Ill (Casals and Webster, Science 97: 246, 1943).

††Neglect of personal hygiene, poverty and errors of diet contribute toward spread of Trachoma.

#In the blood, virus is available for transmission by blood-sucking insects.

[1] Clinical Laboratory Methods and Diagnosis, ed. 4, St. Louis, The C. V. Mosby Co., Vol. II., p. 1698.

MODES OF ENTRY INTO THE BODY

- MUCOUS MEMBRANES
  - RESPIRATORY
    - *Influenza
    - *Common Cold
    - *Measles
    - *Psittacosis
    - *Herpes
    - Variola
    - Varicella
    - Primary atypical pneumonia**
    - Louping ill
    - Poliomyelitis
    - St. Louis encephalitis
    - Lymphocytic choriomeningitis
    - Japanese B encephalitis
    - Australian X disease
    - Foot & mouth disease
    - *Coxsackie
  - ORAL
    - Mumps
  - ALIMENTARY
    - Poliomyelitis
    - Lymphogranuloma venereum
    - St. Louis encephalitis
    - Lymphocytic choriomeningitis
    - Influenza
    - Yellow Fever
    - Psittacosis
    - Foot & mouth disease
    - Coxsackie
  - GENITAL
    - *Lymphogranuloma venereum
    - Warts
    - Inclusion blennorrhea
    - Herpes genitalis
  - CONJUNCTIVA
    - *Trachoma
    - *Inclusion blennorrhea
    - *Lymphogranuloma venereum
    - *Herpes
    - Epidemic keratoconjunctivitis
    - Newcastle disease
- SKIN (abraded and unabraded)
  - *Rabies
  - *B Virus
  - *Warts
  - *Herpes
  - *Yellow fever
  - *Dengue
  - *Sand-Fly fever
  - Variola
  - Varicella
  - Foot & mouth disease
  - Molluscum contagiosum
  - Equine encephalomyelitis
  - Lymphocytic choriomeningitis
- PLACENTA
  - *Measles
  - *Variola
  - *Varicella
  - Lymphogranuloma venereum
  - Lymphocytic choriomeningitis
  - Rift Valley fever

MODES OF ELIMINATION FROM THE BODY

- RESPIRATORY SECRETIONS
  - *Influenza
  - *Common cold
  - *Measles
  - Primary atypical pneumonia**
  - Poliomyelitis
  - Lymphocytic choriomeningitis
  - St. Louis encephalitis
  - Japanese B encephalitis
  - Australian X disease
  - Coxsackie
- SALIVARY SECRETIONS
  - *Rabies
  - *Mumps
  - *B Virus
  - *Herpes
  - Lymphocytic choriomeningitis
- SKIN
  - *Variola
  - *Varicella
  - Warts
  - Foot & mouth disease
- BLOOD#
  - *Yellow fever
  - *Dengue fever
  - *Psittacosis
  - *Lymphocytic choriomeningitis
  - *Sand-Fly fever
  - Forest-Spring encephalitis
- URINE
  - *Lymphocytic choriomeningitis
- FAECES
  - *Poliomyelitis
  - *Psittacosis
  - Influenza
  - Lymphogranuloma venereum
  - *Coxsackie

Within recent years a number of new procedures have appeared in the literature which give promise of simplifying laboratory technic with regard to the diagnosis of virus diseases. Hirst[41] discovered that the allantoic fluid of chick embryos infected with either influenza "A" or "B" virus was capable of agglutinating red blood cells. This phenomenon was utilized to detect influenza virus in throat washings and to measure antibodies in serum. Hirst's studies clearly indicate that the amount of agglutination-inhibiting substances in influenzal antiserum parallels the neutralizing antibody titer more closely than the complement fixing titer. It now appears that this simple in vitro test will be sensitive and specific enough to replace the more cumbersome mouse protection test. The same phenomenon was observed independently by McClelland and Hare[42] who confirmed the observations of Hirst. Nagler[43] has described a similar phenomenon with vaccinia virus. Mumps and certain viral encephalitis viruses give a similar phenomenon.

PREPARATIONS OF SPECIMENS IN THE DIAGNOSIS OF VIRAL DISEASES

If serum, spinal fluid, or other material is mailed to another laboratory, it should be sent in a frozen state. Put it in a sterile ampule or small tube, immerse the glass in a mixture of dry ice and ethyl alcohol. Rotate the vessel to assure proper freezing. Then seal the ampule or the tube, and forward it, packed in dry ice, in a double container.

Several samples of blood and spinal fluid should be collected, at weekly intervals, beginning with the first day when suspicion of a viral disease arises.

Egg inoculations may be carried out if facilities for proper histologic examinations exist. Animal tests are meaningless in diseases other than rabies and variola if proper control strains and sera are not available.

Table 169 shows the most frequently recommended procedures used in the diagnosis of viral diseases.

Virology is a relatively new science. Many methods used in virologic laboratories are not suitable as yet for smaller units. As a general rule, histologic work and complement fixation tests can be performed in nonspecialized laboratories, provided that reliable antigens are available. The method prescribed for use with the particular antigen must be followed to the letter, even more carefully than in work with syphilitic sera. If, for instance, the temperature stated in the outline is 37° C., that does not mean 36.5° or 37.5°, but exactly 37° C. Maintenance of the stock cultures and preparation of the sera for neutralization tests demand so much time, effort, and specialized knowledge that it is usually best to forward such sera for such reactions to a large laboratory which is especially equipped for the work and has properly trained personnel. Most state health laboratories and many university laboratories will have such facilities and will be glad to assist if requested to do so.

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[41] Loc. cit.

[42] Canad. Pub. Health J. 32: 530, 1941.

[43] M. J. Australia 1: 281, 1942.

TABLE 169.—RECOMMENDED LABORATORY PROCEDURES IN VIRAL DISEASES

DISEASE	MATERIAL TO BE SUBMITTED	MICROSCOPIC EXAMINATION	EGG INOCULATION	NEUTRALIZING ANTIBODIES	COMPLEMENT FIXATION	OTHER TESTS
Trachoma, inclusion blennorrhea	Conjunctival scrapings, smears	x				
Psittacosis, ornithosis	Blood Sputum	?	Yolk sac		x	Mouse, intraperitoneal
Lymphogranuloma venereum	Blood				x	Frei skin test
Rabies	Blood Brain	x			x	Mouse, intracerebral
Molluscum contagiosum	Local lesion	x				
Variola	Blood Local lesion	x	Chorio-allantoic membrane		x	Rabbit eye
Herpes	Blood, spinal fluid Local lesion	x	Chorio-allantoic membrane	x	x	
Primary atypical pneumonia						Cold hemagglutination, strep. MG agglutination
Influenza	Blood Nasal washings		Amniotic cavity		x	Hemagglutination inhibition
Mumps	Blood, spinal fluid				x	Hemagglutination inhibition
Equine, Japanese B, St. Louis, and Russian encephalitis	Blood, spinal fluid			x	x	Mouse, intracerebral
Lymphocytic choriomeningitis	Blood, spinal fluid			x	x	Mouse, intracerebral, guinea pig
Dengue, Colorado tick fever, yellow fever	Blood			x		Mouse, intracerebral
Poliomyelitis	Blood Stools			?	?	Monkey
Coxsackie virus	Blood Stools, nasal washings			x	?	Suckling mouse, intraperitoneal
Common cold	Nasal and throat washings					Cell agglutination

Neutralization Tests[1]

Following recovery from a viral or rickettsial disease, blood sera of man and animals generally contain immune substances capable of neutralizing the infectious agent. These protective antibodies, which are highly specific, are demonstrated by adding the serum to infectious material and then inoculating the susceptible host with the mixture. This serves as an indicator of the presence or absence of active agent in the mixture.

The information obtained is useful in diagnosis as well as in estimation of the response of the host to certain vaccines, and the test can be used to identify newly isolated strains of virus.

There is no standard neutralization test. Each method represents a compromise in the selection of various factors which influence the final result: choice of indicator host, route of inoculation, source of virus, type of antiserum, use of constant dilutions of serum with serial dilutions of virus or vice versa, time and temperature of incubation of virus-antiserum mixture, and age of inoculated animals.

The following is an example of a neutralization test in which death or survival of the animal serves to indicate presence or absence of active virus. This is the method recommended by the Neurotropic Virus Disease Commission in 1942 for the diagnosis of epidemic viral encephalitides of North America.

(a) Virus.—

Remove 10 or more brains from mice showing nervous signs.

Grind with sand or Alundum.

Add 10 c.c. of inactivated (56° C. for ½ hour) undiluted rabbit serum for each gram of brain tissue.

Centrifuge at 2,000 r.p.m. for 10 minutes.

Withdraw the supernatant fluid. This is regarded as the 1:10 dilution of the virus.

Titrate part of it immediately. Distribute the rest in 1 c.c. amounts (2 c.c. quantities may be used) in ampules.

Seal the ampules and freeze quickly in a mixture of solid CO_2 and 95% alcohol, and store in a solid CO_2 refrigerator. One or more ampules are thawed and used in each test, the unused portion of the virus being discarded.

(b) Preparation of Virus Dilutions, and Control and Serum-Virus Mixtures.—

Use the 1:10 dilution from the frozen ampule.

Prepare 1:50, 1:500, 1:5,000, etc., up to 1:500,000,000 or 5,000,000,000 dilutions, using 10% rabbit serum in saline as the diluent, and a separate pipette for each dilution.

Add 0.2 c.c. of the selected dilutions to labeled tubes containing 0.2 c.c. of the undiluted unknown serum and also to 0.2 c.c. of a tested negative, undiluted rabbit serum for the control mixtures.

(c) Selection of Critical Dilutions.—

If the preliminary titration has indicated that the LD_{50} titer of the virus to be used is in the range of the 10^{-8} dilution, after incubation at 37° C. for 2 hours, the control dilutions to be tested will be 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}, and the serum dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}. Similarly:

(1) with preliminary LD_{50} titer of virus in the range of 10^{-7}, control dilutions are 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}, while serum dilutions are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6};

(2) with preliminary LD_{50} titer of virus in the range of 10^{-9}, control dilutions are 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, and serum dilutions are 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}.

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[1] Smadel, Joseph E.: Serologic Reactions in Viral and Rickettsial Infections, in Thomas M. Rivers Viral and Rickettsial Infections of Man, ed. 2, 1952, J. B. Lippincott Co. This section is abstracted from the original article by permission of the author, the publisher, and by the National Foundation for Infantile Paralysis, Inc.

These dilutions are selected in order to be within the range of 100% mortality at one end and 100% survival at the other in the control titration, bearing in mind the variations in titer that may be expected with virus suspensions preserved in the frozen state.

If the test is to be used for diagnosis with a virus that maintains its titer well, one may dispense with the lowest dilution in the serum mixtures and use 3 instead of 4 serum-virus dilutions. If the virus preparations have diminished in titer by 1.5 to 2.0 log dilutions during storage, they should be replaced by new materials.

(d) Incubations, Numbers of Mice, and Period of Observation.—

Use 5 mice for each dilution. If it is necessary to limit the number of mice, 4 may be used for each dilution.

Using 0.25 c.c. tuberculin syringes, inject 0.03 c.c. of each dilution intracerebrally into the mice.

The controls and serum-virus mixtures must be incubated in a water bath at 37° C. for 2 hours and then placed in an ice bath until ready to use.

Inject the highest dilutions first (10^{-10}, 10^{-9}, etc.). The control mixtures should be injected last.

Observe the mice for at least 10 days in the case of Western and Eastern equine encephalomyelitis and for at least 14 days in the case of St. Louis encephalitis virus. Report deaths when they occur. All deaths within 24 hours after inoulation must be regarded as due to traumatic or nonviral causes, and in the case of St. Louis encephalitis virus, all deaths during the first 3 days must be regarded as nonspecific.

Example

TABLE 170

	FINAL DILUTION OF VIRUS						LD$_{50}$ TITER LOG OF DILUTION	NEUTRALIZATION INDEX	RESULT
MIXTURE	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}			
Control	---	---	5/5	4/5	3/5	0/5	8.0		
Serum A	5/5	5/5	5/5	3/5	---	---	7.0+	<10	Negative
Serum B	5/5	5/5	3/5	2/5	---	---	6.5	32	Equivocal
Serum C	5/5	4/5	3/5	0/5	---	---	6.0	100	Positive

Fraction 3/5 indicates that 5 mice were inoculated and in 3 death occurred as a result of the virus infection.

TABLE 171.—PROCEDURE USED IN CALCULATING LD$_{50}$ TITER*

CONTROL

			ACCUMULATION TOTALS			
	DIED	SURVIVED	DIED	SURVIVED	DEATHS	% MORTALITY
10^{-6}—5/5	5	0	12	0	12/12	100.0
10^{-7}—4/5	4	1	7	1	7/8	87.5
10^{-8}—3/5	↑ 3	2 ↓	3	3	3/6	50.0
10^{-9}—0/5	0	5	0	8	0/8	0

Therefore LD$_{50}$ titer, log of dilution = 8.0.

Arrows indicate direction of addition for accumulation totals.

*The Reed-Muench formula will be used even in the absence of a zero mortality end point or of 2 dilutions below the 50 per cent or more mortality range, with the clear understanding that the results are not absolutely accurate. This is the best available procedure for a standard method of expressing the results under circumstances which make it impractical to increase the number of dilutions to cover the strict requirements of the Reed-Muench formula.

SERUM B

			ACCUMULATION TOTALS			
	DIED	SURVIVED	DIED	SURVIVED	DEATHS	% MORTALITY
10^{-4}—5/5	5	0	15	0	15/15	100.0
10^{-5}—5/5	5	0	10	0	10/10	100.0
10^{-6}—3/5	3	2	5	2	5/7	71.4
10^{-7}—2/5	2	3	2	5	2/7	28.6

$$\frac{\text{\% mortality above 50\%} - 50\%}{\text{\% mortality above 50\%} - \text{\%mortality below 50\%}} = \text{factor or proportionate distance or}$$

$$\frac{71.4\% - 50\%}{71.4\% - 28.6\%} = 0.5$$

When tenfold dilutions are used, LD_{50} titer = log of dilution above 50% + factor or in this case = 6.0 + 0.5 = 6.5.

Calculation of Neutralization Index for Serum B

Control titer log of dilution = 8.0
minus
Serum B titer log of dilution = 6.5
Neutralization index log = 1.5
Antilog of 1.5 = 31.6 or 32; therefore neutralization index = 32.

The Commission regarded a neutralization index of 1 to 9 as negative, of 10 to 49 as equivocal, and of 50 or more as positive.

Finding a neutralizing antibody against a virus in a single specimen of serum usually cannot be regarded as proof that the recent illness was caused by the virus in question. Neutralizing antibodies which appear following many infections persist for long periods of time. The picture is further complicated by the occurrence of neutralizing substances in sera of persons who have suffered unrecognized disease in the form of inapparent or atypical infection, or who have been vaccinated with active or inactive virus. Therefore, the presence of a specific neutralizing antibody can be regarded as evidence of previous experience with the agent but no date can be given for the episode.

For this reason, two samples of serum must be taken for testing: the first is taken early in the disease when antibodies are absent or at a low level, and the second at an appropriate time during convalescence.

Hemagglutinin Inhibition Test

The agglutination of erythrocytes by microbial agents, called "Hirst's phenomenon," is a valuable aid in investigating and in diagnosing a number of viral diseases. The test given here as an example is that used for influenza.

Materials Needed.—

0.85% sodium chloride in freshly distilled water, the **Saline solution.**

Type O human red blood cells, either fresh or preserved in Alsever's solution.

Standard dried type A (PR8), type A′ (FM1), and type B (Lee) antigen.

Standard dried chicken antisera against each of the viruses mentioned above.

Kahn tubes, 12 by 75 mm., with evenly rounded bottoms.

Racks for Kahn tubes, flat-bottom type without depressions.

1 c.c. serologic **pipettes,** graduated in 0.01 c.c., and **5 c.c. pipettes,** graduated in 0.1 c.c.

Glassware must be chemically clean. Traces of acid or alkali interfere with the test. Kahn tubes which do not have evenly rounded bottoms should be discarded. For influenza work, reserve specially selected and clean tubes.

Standard Antigens.—

Antigens are prepared according to the method of Hilleman, Buescher, and Smadel.[*]

Inoculate 9- or 10-day embryonated eggs from chickens free of *Salmonella pullorum* and Newcastle disease, by way of the allantoic cavity, with 0.4 c.c. amounts of a dilution of the appropriate seed virus, usually 10^{-4} to 10^{-5}, which has been found in preliminary titrations to produce chorio-allantoic fluids with hemagglutinin titers of 1:320 or greater.

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[*] See footnote, page 1752.

Incubate the eggs at 35° C. for 44 to 48 hours.

Candle the eggs and select those in which the embryos are still alive.

Chill these overnight in a refrigerator at 4° C.

Harvest the allantoic fluids aseptically and pool.

Test for sterility.

Add Merthiolate to a final concentration of 1:10,000.

Distribute these pooled fluids, or antigens, in 5 c.c. amounts in appropriate containers, and lyophilize in a chamber or manifold drier so that a final moisture content of less than 1% is assured.

When properly sealed to exclude moisture, such antigens are stable during storage for at least three years at refrigerator temperature.

Control Antisera.—

Control antisera are prepared in chickens.

Select roosters weighing 6 to 8 pounds.

Inject both intravenously and intraperitoneally with 5 c.c. amounts of the appropriate freshly harvested allantoic fluids having hemagglutinin titers of 1:320 or greater.

After 10 days, bleed the roosters to the point of exsanguination by cardiac puncture.

Allow the blood to clot.

Cut the clots into small pieces.

Store overnight at 4° C., and 4 or 5 hours at room temperature the following day to facilitate contraction of the clot.

Centrifuge and remove the serum.

Inactivate at 56° C. for one-half hour.

Dispense in 0.5 c.c. amounts in ampules for drying by the chamber or manifold method.

Satisfactory sera are those which (1) have inhibitory titers of 1:800 or greater when tested with the homologous antigen and of not more than 1:100 when tested with the two heterologous antigens; and (2) are obtained from roosters which possess no viral inhibitor prior to immunization.

Properly dried and sealed antisera are stable for at least several years.

Titration of Antigens.—

Before use, rehydrate the standard antigen aseptically with 5 c.c. of distilled water.

Remove a sample of this stock material for the day's work, and store the remainder in a stoppered bottle at 4° C. or preferably at –20° C.

Dilute the sample with 10 parts of physiologic saline.

Allow to stand for 1 hour at room temperature, to facilitate elution of virus from the small amount of particulate matter in the preparation.

Using Kahn tubes, mix 0.5 c.c. volumes of serial twofold dilutions of antigen (1:20 to 1:5,120) in physiologic saline with 0.5 c.c. volumes of a 0.5% suspension of human group O red cells.

Shake thoroughly.

Read the pattern of the sedimented cells after 60 to 70 minutes at room temperature. The tubes must not be disturbed during this period.

The last tube showing hemagglutination is the end point of the titration.

The diagnostic test for influenza antibodies employs 4 units of virus in 0.25 c.c. In the above titration, the agent is contained in 0.5 c.c.

Therefore, the concentration required for the serum test is 8 times that represented as the end point of the virus titer.

Thus, if the last tube showing agglutination contained 0.5 c.c. of a 1:320 dilution, then 8 times this concentration, or a 1:40 dilution, that is, 1:2 of the day's stock or 1:40 of rehydrated antigen, would contain 4 units in a 0.25 c.c. volume.

Erythrocyte Suspension.—

Use human blood, group O, either fresh or preserved not longer than 1 month in Alsever's solution.

Wash three times in saline solution.

For the final centrifugation, place the suspension in a 15 c.c. graduated tube.

Centrifuge at 1,500 r.p.m. for 10 minutes in a centrifuge having a horizontal head with a radius of 5½ inches from axis to trunnion slot.

Withdraw the supernatant fluid without disturbing the packed cells.

Read the volume of cells.

Add enough saline solution to make a 0.5% suspension.

The suspension is usable only on the day of preparation.

Diagnostic Agglutination-Inhibition Test.—

Two serum specimens from each patient must be tested simultaneously with all three types of antigen. The first of these, or the acute phase, is obtained within 2 or 3 days after onset of illness; the second or convalescent serum 10 to 14 days after onset of illness.

Inactivate 0.3 c.c. amounts of the sera to be tested, at 56° C. for 30 minutes.

Dilute each serum 1:8 by adding 2.1 c.c. of saline solution to each.

Use three rows of 10 Kahn tubes in a rack for each serum.

Place in tubes 2 to 10 in the front row 0.75 c.c. of saline.

Leave tube 1 in the front row and all the tubes in the middle and back rows empty.

Place in the first tube of each row 0.25 c.c. of inactivated 1:8 dilution of serum.

Place 0.75 c.c. of the same dilution of serum (1:8) in tube 2 of the front row.

Mix tube 2, front row, and transfer 0.25 c.c. amounts to tubes 2 of the middle and back rows.

Transfer 0.75 c.c. from tube 2, front row, to tube 3, front row.

Mix contents of tube 3, and place 0.25 c.c. amounts in the tubes 3 of the middle and back rows, and 0.75 c.c. in tube 4 of the front row.

Continue in this manner throughout the 10 tubes.

There are now 3 identical series of twofold dilutions, with each tube containing 0.25 c.c. of the serum in dilutions ranging from 1:8 to 1:4096.

Add 0.25 c.c. of properly diluted PR8 test antigen (4 units) to each tube in the first row.

Add 0.25 c.c. of FM1 antigen to each tube in the second row.

Add 0.25 c.c. of Lee antigen to each tube in the third row.

Shake thoroughly.

Add 0.5 c.c. of 0.5% erythrocyte suspension to the serum-antigen mixtures, in each tube.

Shake thoroughly.

Leave the racks undisturbed at room temperature for 60 minutes. Read.

The titer of the serum is the highest dilution which inhibits hemagglutination.

Example: If inhibition is observed in tubes 1 to 7 of the front row, and 1 through 3 in the middle and back rows, the titer would be 1:512 with PR8 and 1:32 with FM1 and Lee antigens.

Controls.—

Standard Antisera:

Antisera from roosters immunized against the PR8, FM1, and Lee strains of influenza virus are used for controls, and are included in each day's tests.

Add 5 c.c. of sterile distilled water containing 1:10,000 crystalline Merthiolate to a specimen of lyophilized inactivated serum, making a 1:10 dilution of the original serum.

Store this stock solution at 4° C. in a sterile, tightly stoppered container.

Remove 0.3 c.c. of the stock for the day's work.

Mix with 2.7 c.c. of saline solution, making a 1:100 dilution.

Perform agglutination-inhibition tests with the standard antisera in the same manner as for unknown sera, except that tubes No. 1 contain 1:100 dilution instead of 1:8.

These controls serve to confirm the identity of the antigen used, and assist in correlating the results obtained in tests performed on different days.

Although the titer of a given serum is inversely affected by changes in the concentration of virus used in the test, the titer of a positive control serum is more regularly reproducible than that of an antigen preparation since it is changed little by minor differences, a unit or more, in the amount of antigen employed. The end point for a given standard antigen should not vary more than a single twofold dilution from that obtained in previous tests.

Retitration of Test Antigen:

A portion of the diluted stock antigen containing an estimated 4 agglutinating units in 0.25 c.c. and used in the agglutination-inhibition test must be retitrated to determine its potency. This is done in the same manner as the initial antigen titration after rehydration except that tube No. 1 receives 0.5 c.c. of the diluted stock. Complete agglutination should occur in the first 4 tubes of the titration.

Interpretation.—

Report results in terms of the titers of each of the paired sera obtained with each antigen.

A fourfold or greater increase in titer with any one of the antigens during convalescence is considered of diagnostic significance.

A rise in titer to both type A and type B virus should be suspected as due to recent vaccination, and a thorough investigation of this possibility should be made.

Concurrent infection in a single individual with both type A and type B influenza virus is a rare occurrence.

Cold Hemagglutination Test in Diagnosis of Primary Atypical Pneumonia

That the cells of an individual may be agglutinated by his own serum has been observed for many years. It was also noted that clumping of these cells was more marked when the blood was chilled; moreover, that this phenomenon seldom occurred at body temperature. In 1943, Peterson, Ham, and Finland first noted that this agglutination reaction was found in cases of primary atypical pneumonia. Numerous contributions have been made since that time, among them that of Humphrey.[1] In this series 93 per cent had positive reactions. In a series of 80 controls, representing patients with upper respiratory and pulmonary diseases, there was no strong reaction. The first positive reactions were noted within 4 to 8 days after the onset, but in some instances the reaction might be delayed until the early part of the third week. The maximum titer usually occurred between the sixteenth and twenty-third days of illness, and became weak or negative between the thirty-fifth and forty-fifth days. There is very little relationship between the titer and the fever, although some parallelism exists between the test, the sedimentation rate, and the x-ray findings.

The advantages of the test lie in the fact that a definite diagnosis can often be made by its use. In certain instances the test may prove to be a means whereby a simple and economic procedure may supplant the more costly chest x-ray films. A disadvantage lies in the fact that the test is not consistently positive in the first week of the illness but it must be remembered that most of the agglutination reactions for other diseases are positive no earlier.

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[1] U. S. Nav. M. Bull. 43: 1117, 1944.

Technic.—The method described by Humphrey may be accepted as a standardized procedure.

Place 6 drops of blood in a centrifuge tube containing 10 c.c. of 2.5 per cent sodium citrate solution. Mix by inverting the tube. To insure uniformity use a 21-gauge needle with the bevel held downward while the 6 drops from the syringe are delivered.

Place 4 to 6 c.c. of noncitrated blood in a small test tube and permit to clot. If the serum does not separate readily from the clot, centrifuge the specimen.

Centrifuge the cells for 15 minutes at 2,000 r.p.m., pour off the supernatant citrate, and resuspend the packed cells in 2 c.c. of 0.85% saline solution.

Place 0.75 c.c. of physiologic saline solution in a small test tube and add 0.25 c.c. of the serum to make a 1:4 dilution. Add to this 0.1 c.c. of the cell suspension.

Place this small test tube in a rack which is immersed in water and ice, and then place the rack and its container in an ice chest or refrigerator. Under ordinary circumstances the temperature will remain between 1° and 4° C. for hours, but it should be checked by a thermometer immersed in the ice and water mixture.

Allow the tube to remain in the refrigerator overnight and then read. If agglutination is present it will disappear in a few minutes, or at the most within an hour, at room temperature. When the mixture is replaced in the ice bath, it will reagglutinate within a few hours. This reversibility is characteristic and should be employed in doubtful cases.

To Determine Titer.—Make further dilutions by setting up a series of 10 test tubes, placing 1.5 c.c. of 0.85% saline solution in the first tube, and 1 c.c. in each of the remaining nine.

Pipette 0.5 c.c. of the serum into the first tube of the series, transfer 1 c.c., after mixing, to the second, and so on down the line of tubes, discarding the last c.c. from the tenth tube. Dilutions of serum are 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048.

Add 0.1 c.c. of the patient's own cell suspension to each tube, and place the tube in the ice bath overnight.

Reading the Agglutination Reaction.—Usually the result is definitely positive or negative, but a standard method of agitation should be employed. The tube should be inverted slowly four times, or flicked with the finger a definite number of times before reading. Placing the racks in the Kahn shaker, set at the usual speed, requires approximately 15 seconds, but this refinement is not considered necessary after some practice has been gained in reading.

If the reaction is 3 plus or 4 plus, the heavy clumps float in a clear fluid and quickly settle to the bottom. If the red cells form a solid clump or disk, it is definitely a 4 plus reaction. If the clumps are fine and granular on agitation, but the background is still a clear solution, it should be considered a 2 plus reaction; if the background is faintly pink or pearly it is a 1 plus reaction. Negative reactions show the pink iridescence indicative of individual cell dispersion. If this pearly or nacreous sheen is present in any degree, even in the presence of clumps, it should be considered a negative reaction.

Cultivation of Viruses on the Chorio-Allantois of the Developing Chick

This is discussed fully on pages 1473 and 1745.

THE BACTERIOPHAGE

The Isolation and Cultivation of the Bacteriophage

Explanation of Terms Used.—

A bacteriophage, according to d'Herelle, is an ultramicroscopic living corpuscle which derives its nourishment entirely from the bodies of living bacteria. In its parasitic action against the bacteria, the bacteriophage actually dissolves the bacteria on which it is living. The bacteriophage supposedly multiplies in the bodies of bacteria, just as bacteria multiply in culture media.

The bacteriophage is small enough to pass through the pores of a bacterial filter, such as the Berkefeld filter.

The process of this multiplication at the expense of the bacterial bodies is called **bacteriophagy**. The bacterium acted upon is said to be undergoing the process of bacteriophagy.

Bacteria are said to exert a **resistance** to bacteriophagy in the same manner as an animal body resists bacterial invasion of its tissues.

Virulence of bacteriophages against bacteria may be increased or diminished, just as virulence of bacteria may be changed. By passing bacteriophages successively through bacteria of increased resistance, the degree of virulence of the bacteriophage (produced by these successive passages) increases. Similarly, by placing the bacteriophages under adverse environmental conditions, the virulence may be attenuated.

The bacteriophage corpuscles are found normally in the intestinal tract, and are capable of being isolated from fecal matter. They are also found in secretions and excretions from other parts of the body.

The effect of bacteriophagy on a liquid medium is to render the cloudy medium clear. The effect on solid mediums is similar; that is, ''plaques,'' or bare areas, on the agar may be seen interspersed between the solid bacterial growth. These plaques represent ''colonies'' of bacteriophage corpuscles. After reaching maturity, the plaques do not increase in size.

Isolation of the Bacteriophage*

The isolation of the bacteriophage may be undertaken under one or another of the following circumstances:

1. The bacteriophage may be sought in a sterile fluid, for example, in normal blood, or in an organic fluid collected aseptically. With such no treatment is necessary.

2. The material to be examined may be a clear, but not sterile, liquid. With this, filtration will eliminate the bacteria while the bacteriophage passes through into the filtrate.

3. The material may show a homogeneous turbidity; such as a bacterial culture. Here, direct filtration results in an early occlusion of the pores of the filter candle. Thus, it is desirable to resort to a preliminary filtration. The following method of treatment is most satisfactory.

Provide a funnel with a folded filter paper sufficiently large to receive at one time the entire volume to be filtered.

Fill the funnel with water to which has been added a small amount of infusorial earth.

When the water has passed through, the paper is left coated with a thin layer of the infusorial earth, thus rendering the paper less permeable.

Through this, the material to be examined is filtered, prior to filtration through the candle.

4. The material may be a fluid holding in suspension organic particles, or it may be matter more or less solid in nature. This is the type of substance most frequently examined; such as, fecal material, more or less fluid, pasty, or solid; or excreta admixed to a greater or lesser degree with earth, organic debris, etc. In such case it is necessary to disintegrate as completely as possible the material to be examined. To effect such disintegration the simplest procedure consists in carefully suspending the material in bouillon, about 5 gm. to 50 c.c. of the medium, and incubating this suspension at 37° C. for from 12 to 18 hours. The bacterial fermentations which ensue, because of the divers organisms introduced into the medium, lead to a sufficient disintegration. Upon removal from the incubator the material may be treated, as indicated above, by filtration through infusorial earth and a candle.

If the material under examination contains the bacteriophage and has been subjected to filtration, the bacteriophage will be found in the filtrate.

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*Condensed from d'Herelle: The Bacteriophage and Its Behavior, translated by George H. Smith: Williams and Wilkins, Baltimore, Md., 1926, pages 37 ff.

Method of Isolation of Bacteriophages

Bacteriophages pass through Berkefeld or Seitz filters and, therefore, may be obtained by filtration of the material from which they are obtained.

They also resist heat at 60° C. If, therefore, there are no spore-bearers present, bacteriophages may be isolated by heating the material from which they are to be derived at 60° C. long enough to kill the vegetative forms of the bacteria.

Assume that the bacteriophage to be isolated is the one which is active against the typhoid bacillus. Obtain the feces from a convalescent typhoid case.

Suspend the feces in broth M-1, about 5 gm. to 50 c.c. medium.

Incubate at 37° C. for 12 to 18 hours.

Filter through infusorial earth, then a bacterial filter.

The bacteriophage, if present, will be found in the filtrate.

Technic of Cultivation

A. **In a Liquid Medium**: Make a heavy inoculation of the selected bacteria in a tube containing 10 c.c. sterile nutrient broth M-2. The broth becomes turbid.

Incubate about 4 hours at 37° C.

Add to this suspension of living bacteria the desired quantity of bacteriophage. The bacteriophage is either derived in the manner described above, or may be purchased from various supply houses. When an active race of bacteriophage is used, 0.05 c.c., or 1 drop, is sufficient quantity for the inoculation. If the strain is new, or unknown, use 1 c.c. of the filtrate from the Berkefeld preparation.

Replace the mixture of bacterial suspension and bacteriophage in the incubator.

Observe the results at the end of each hour, until the medium has become clear. At this point, bacteriophagy is complete. Often bacteriophagy is complete at the end of three hours' incubation.

Quite frequently, after bacteriophagy has apparently taken place to such an extent that all bacteria in the suspension seem to have been dissolved, further incubation leads to the development of secondary cultures. If bacteriophagy is absolutely complete, the tube in which the phenomenon occurred will remain clear indefinitely. In order to prevent the development of secondary cultures, which may obscure the observation of bacteriophagy, as soon as bacteriophagy has taken place (seen by the clearing of the medium), filter the culture through a Berkefeld filter. Transplant the filtrate to cultures of the same species of bacteria as used in the original culture. **Serial passages** of bacteriophage races may be made through suspensions of bacteria in nutrient broth M-2 from an agar culture, as follows:

Inoculate this turbid suspension with the bacteriophage to be cultivated.

Place in an incubator at 37° C. until the suspension becomes clear.

Make another suspension of bacteria in nutrient broth, following the instructions in the preceding paragraph.

Pick up a loopful of the clarified broth (which has just been taken from the incubator), and place in the tube of suspended bacteria.

Place this tube in the incubator at 37° C. until it clears. Repeat this process as often as desired.

The bacteria in each successive suspension should be completely dissolved. If there is possibility that any bacteria remain in the suspension undissolved, filter through a Berkefeld filter. In transferring bacteriophage suspensions, unusual care must be taken to eliminate possibility of air contamination.

In preparing suspensions of bacteriophage corpuscles for therapeutic use, the cultures must be filtered through a Berkéfeld or Seitz filter. It is advisable to filter twice to insure sterility.

B. **In a Solid Medium**: Make a heavy inoculation of 10 c.c. of sterile nutrient broth with bacteria taken from an agar culture. The suspension should be made in such way that it is markedly turbid.

Add a drop of bacteriophage suspension. (If a particularly active bacteriophage suspension is used, it must be diluted many hundreds of times before inoculating the broth. Saline is used as the diluent.)

Mix thoroughly.

Remove a drop of this mixture of suspended bacteria and bacteriophage corpuscles with a sterile dropper with a rubber bulb attached, or a sterile pipette.

Place on the surface of an agar plate. The agar in the plate should not be in too thin a layer, since the products of bacteriophage growth must be absorbed through the culture medium to enable the corpuscles to grow to their fullest extent.

Allow the drop to spread over the surface of the agar plate so that all portions of the plate are covered by the drop.

Incubate at 37° C. overnight.

The bacteriophage present will be seen in isolated glassy-appearing clear areas, or "plaques," over the surface of the agar plate, which is covered, except in the areas of the plaques, by the bacterial growth. If too heavy a suspension of bacteriophage corpuscles has been used in making the inoculation, the entire surface of the agar will present the glassy clear appearance of the isolated bacteriophage patches. In other words, it will present the appearance of a sterile uninoculated plate of agar.

By touching one of the plaques, and inoculating a suspension of bacteria with the bacteriophage corpuscles thus picked up (although the loop will appear as though there is nothing present on it), the bacteriophage may be successively transplanted over an unlimited period of time.

If the layer of bacterial growth is very thick, the plaques made by the growth of the bacteriophage will be correspondingly small.

Another method of inoculating solid media is to make a surface inoculation of the medium with bacteria so that the entire plate has been touched with the inoculating material. Dip a sterile rod into a suspension of bacteriophage corpuscles in broth, and streak with this rod across the surface of the plate.

Incubate the plate at 37° C. overnight.

The bacteriophage will grow in a solid plaque, or clear area, over the portion of agar touched by the rod. In other words, there will be a solid bacterial growth on the plate except in the areas touched by the rod. These areas will appear as if they are sterile.

A Procedure for the Preparation of Autogenous Bacteriophage for Therapy

(SUGGESTED BY NORMAN R. GOLDSMITH, LANCASTER, PENNSYLVANIA)

1. Loops directly from the infected area or material are streaked on an agar plate so as to obtain isolated colonies. A tube of broth is also inoculated. If the organism has been in pure culture, this tube will suffice; if not, a pure culture will be obtained from the plate. If there is more than one type colony, a separate bacteriophage must be prepared for each one, or as many as possible, since it is rare to find a single phage race potent against two organisms from the same source. A working diagnosis of the organism is determined from a Gram-stained smear, the appropriate races of stock bacteriophage being then selected.

2. A tube of pure culture is then allowed to incubate until it is markedly cloudy, but not over twenty-four hours. It is important to remember that the phenomenon of bacteriophage lysis occurs only with young, actively growing organisms.

3. An odd number of test tubes containing about 5 c.c. of medium each is then set up. To each two tubes, a single race of the stock bacteriophage is added, a different race to each pair of tubes, the quantity of phage being about five drops. All work is done with oven-sterilized, cotton-plugged pipettes. Phage lysis is such a variable matter that extreme accuracy, such as the use of absolutely uniform pipettes so as to obtain exactly equal-sized drops, is not a necessity. An approximation of the proportions given here should produce satisfactory results. The tendency is to use too great a quantity of phage. This often results in an impaired permanency of lysis.

4. One drop of the pure culture of bacteria is then added to each of the tubes containing phage; and to the remaining odd tube which serves, first, as a control of bacterial growth uninfluenced by phage, and, second, as the source of the pure culture for the next generation.

5. The tubes are then incubated until the above control tube is very definitely cloudy. A comparison with the tubes containing phage is then made. Certain pairs of tubes will be found to be less turbid than the control. The degree of lessened turbidity will vary greatly from almost complete clarity to a turbidity indistinguishable from the control. The tubes which are less turbid than the control are those in which some degree of phage action is taking place.

6. If no tubes appear to be less turbid, either the strain of bacteria is resistant to all the races of bacteriophage tested against it, or the degree of lysis is too small to be noticed by the ordinary eye. Ability at detecting extremely slight degrees of turbidity variation is achieved after considerable experience. A method of determining whether phage is present, which at times is effective, is to streak on agar a loop from the phage-inoculated tube beside one from the control tube. A dissimilarity between the two streaks, with a moth-eaten appearance on the streak from the phage-inoculated tube, is indicative of the presence of bacteriophage plaques. A more potent phage, sufficient to produce macroscopic clearing, may then be produced by the serial filtration method to be described. It must be remembered that certain strains of bacteria are absolutely resistant to any races of bacteriophage known today, and a certain percentage of failures is unavoidable.

7. The serial filtration method increases the specificity and potency of a given bacteriophage for a given organism. (There are a number of types of filter on the market; the most satisfactory in our hands has been the Chamberland-Pasteur, the technic being similar to that described in d'Herelle's ''The Bacteriophage and Its Behavior,'' 1926, p. 22. Many laboratories use the Berkefeld or others with excellent results.)

From each pair of partially lysed tubes, one is taken and filtered. The remaining tube is kept for observation of lysis permanency. The tubes are filtered through the same filter, thus producing a pooled filtrate of the most virulent races of phage for that particular organism.

8. Three tubes are now set up. To two of these, five drops of the filtrate are added. One drop of the previous control tube, provided it is not too old, is now added to each of the tubes containing the filtrate and to the remaining tube which in its turn becomes the untreated bacterial control. The remainder of the filtrate is put in a sterile tube and serves as an automatic check of phage sterility or as a reserve in the event of some unforeseen difficulty or failure.

9. The tubes are now incubated together. This time, the contrast between the phage-treated tubes and the control should be greater, and lysis should last for a longer period. Again, one of the lysed tubes is filtered, and the other kept for permanency observation. There is no arbitrary time rule when to filter the tubes. The period varies with each organism. In general, twenty-four hours is too long when first developing a phage, and filtrations should be made between four and eight hours after planting, else a secondary growth will appear in the tubes temporarily cleared, and the process will have to be repeated.

10. Another set-up of three tubes is made. The filtrate from step 9 is added in 5-drop amounts to two tubes; one drop of the previous control is added to them and to the remaining tube, which, in its turn, is to serve as bacterial control; the remainder of the filtrate is stored and tested for sterility. Tubes are then incubated.

11. This process of repeated filtrations is continued until a filtrate is obtained which produces a lysis persisting for twenty-four hours or more. This may necessitate as many as 10 separate filtrations, but three to six is the average. (Obviously, in emergency work this ideal cannot be obtained, but when time permits, better therapeutic results will be obtained with a highly potent phage rather than a weak one.)

12. A large volume can now be prepared. Add 2 c.c. of the last filtrate to 100 c.c. of media in an Erlenmeyer or Florence flask. Then add about 21 drops of the previous bacterial control. Tubes are also prepared for a new bacterial control and for a retest of lysing permanency using similar proportions. At times, a cleared flask will again become cloudy. Do not discard this immediately unless it contains an obvious contamination. Often it will clear again, and a filtrate of this second clearing usually yields an extremely potent phage. If the flask does not reclear, it should be discarded and another flask planted.

13. The contents of the flask, if clear, are filtered and the filtrate is transferred with a sterile 10 c.c. pipette into sterile tubes in 10 c.c. amounts. This is for local therapy. Other 10 c.c. quantities are put into rubber-stoppered vaccine bottles for intradermal, subutaneous, or intravenous use, or shipment. The tubes and ampules are incubated in their entirety for twenty-four hours. Any which are not absolutely crystal clear are discarded.

14. Simultaneously, the titer of the bacteriophage may be determined. Then tubes containing exactly 4.5 c.c. of media are set up. To the first of these, exactly 0.5 c.c. of the previous filtrate is added and mixed. This gives a 1:10 dilution. Half a c.c. of this is transferred to the next tube, and so on, the dilution in the tenth tube being 1:10,000,000,000. To each of the tubes, and a control tube, one drop of the previous control is added. Observations as to what dilution lysis is present and permanent are made. An agar slant of the organism is made in order to keep the specific strain in the event of more autogenous phage being required.

15. The tubes which have been kept as a test of lysing permanency, if still clear, are now pooled and filtered together. This usually gives an extremely efficient phage. It is added to our stock of phage races and is used in testing new strains of bacteria.

Criteria for supplying bacteriophage: If the tubes are sterile, if cultures keep clear for 24 hours, and if the titer against the organism is at least $1:1 \times 10^{-7}$.

CHAPTER XI

MEDICAL MYCOLOGY

CHESTER W. EMMONS, PH.D.*

The lowest class of the plant kingdom, the Thallophyta, includes the Algae, which possess chlorophyll and therefore are autotrophic, and the Fungi, which lack chlorophyll and therefore are saprophytic or are parasitic on other plants or on animals. The Fungi include Schizomycetes (bacteria), Myxomycetes (slime fungi), and Eumycetes (true fungi).

The true fungi present a great variety of forms, including simple, unicellular, spherical, or elliptical cells which reproduce by budding (as in the yeasts), multicellular but microscopic organisms consisting of tubular, septate filaments which bear spores in various ways upon specialized hyphae or in complex fruiting structures (as in the molds), and those fungi which produce large, conspicuous reproductive structures (the mushrooms, toadstools, bracket fungi, etc.).

Numbering some 200,000 species, the true fungi are classified among Phycomycetes, Ascomycetes, Basidiomycetes, and Hyphomycetes. The Hyphomycetes are also called "fungi imperfecti" because they have only a vegetative or asexual type of reproduction without a known antecedent nuclear fusion. When study of the life history or chance observation reveals a more definitive type of reproduction in a member of this group, it is transferred to one of the three other groups. Most of the fungi pathogenic for man remain in this group, the life histories of which are incomplete or are incompletely known. Martin[1] has written a useful outline on classification of the fungi and the student may refer to Bessey's[2] authoritative text for a more detailed exposition of the principles of general mycology. An excellent handbook in medical mycology has been prepared by Conant and associates.[3]

The following **glossary** may be useful. A complete and authoritative glossary is to be found in Ainsworth and Bisby's *Dictionary of the Fungi.*[4]

Hypha.—A tubular, branching filament divided by cross walls (septa) into cylindrical cells. In some fungi septa are very infrequent.

Mycelium.—The mass of branching, frequently anastomosing hyphae which make up a colony of a fungus.

Conidium.—A spore borne externally, either terminally or laterally, on a hypha which may be morphologically specialized (a conidiophore). Conidia may be borne singly, in clusters, or in chains, they may be unicellular or multicellular, and they vary greatly in size, shape, and color.

Macroconidium.—The larger of two types of conidia produced by a given fungus (e.g., the "fuseaux" of the dermatophytes).

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*From the U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute (Laboratory of Infectious Diseases), Bethesda, Md.

[1] Martin, G. W.: Outline of the Fungi, Univ. of Iowa Studies 28: 1-64. 1941.

[2] Bessey, E. A.: Morphology and Taxonomy of Fungi, Philadelphia, 1950. The Blakiston Co.

[3] Conant, N. F., Smith, D. T., Baker, R. D., Callaway, J. L., and Martin, D. S.: Manual of Clinical Mycology, ed. 2, Philadelphia, 1954, W. B. Saunders Co.

[4] Ainsworth, G. C., and Bisby, G. R.: A Dictionary of the Fungi, Kew, 1950, The Commonwealth Mycological Institute.

Endospore.—A spore abstricted within a tubular mother cell.

Sporangiospore.—A spore borne by "progressive cleavage" within a mother cell, the sporangium.

Arthrospore.—A spore formed by septation of a hypha. These may remain cylindrical or may round up into barrel-shaped or even elliptical cells.

Chlamydospore.—A thick-walled, intercalary or terminal resting spore.

Blastospore.—A term sometimes applied to the budding cells of a yeast, which are actually not spores but vegetative cells.

Conidiophore.—A specialized hypha which bears conidia.

Ascospore.—A spore formed by "free cell formation" within a mother cell (ascus), following a nuclear fusion and a subsequent reduction division. Typically there are 8 ascospores per ascus.

Basidiospore.—A spore borne externally on a basidium, following a nuclear fusion and subsequent reduction division. Typically there are 4 basidiospores per basidium.

Zygospore.—A spore formed by the fusion of two specialized hyphal tips with subsequent nuclear fusions.

Characteristics of Mycoses

Mycoses may be classified as superficial or systemic, although this is not a rigid scientific classification and many of the systemic mycoses commonly have skin manifestations.

The superficial mycoses include the dermatophytoses (ringworm, tinea, athlete's foot), a group of infections involving the skin and its appendages and caused by a group of related fungi. Thrush is another predominantly superficial mycosis involving the mucous membranes but it sometimes becomes systemic. Some mycoses invade the subcutaneous tissues (chromoblastomycosis) while others are truly systemic and invade any tissue of the body (coccidioidomycosis, blastomycosis, histoplasmosis). To varying degrees most of the pathogenic fungi are dimorphic, growing in one form in tissue or body fluids and with very different and usually richer morphology in culture. Therefore direct microscopic examination of exudates or sections and of cultures is essential in the identification of a fungus.

Saprophytism; Laboratory Infections

Recent research studies have emphasized the saprophytic sources of infection in the mycoses. An understanding of this is pertinent to the laboratory study of the pathogenic fungi. Contagion plays a minor or negligible role in the systemic mycoses and the infecting fungus is usually acquired from a saprophytic source in the environment of the patient rather than from another person. It follows that when a fungus is grown in the laboratory it reverts to its natural saprophytic state, produces large numbers of infectious spores, and becomes a potential hazard unless it is handled with caution. Laboratory infections with Coccidioides are especially frequent. Fortunately most of these have been benign, but clinical severity of a laboratory infection appears to be related to intensity of exposure. It therefore is important that the mycologist in a diagnostic laboratory observe certain precautions. Manipulations of cultures should be carried on so far as possible under a hood. Cultures should be maintained on agar slants rather than on Petri dishes, but if cultures must be made in Petri dishes the fungi should be transferred while colonies are young. The opening of an old plate culture endangers laboratory personnel if a pathogen is present and unnecessarily contaminates the laboratory if miscellaneous harmless molds are present.

HISTOPATHOLOGIC METHODS

Fungi are poorly differentiated in tissues by the routine histopathologic stains, and when present in small numbers may be overlooked. Staining schedules utilizing the Schiff reagent clearly demonstrate fungi, although mucin and other nonfungal elements in some tissues also stain. The Bauer[1] and Hotchkiss-McMannus technics are useful applications of this method to the study of mycotic lesions. Kligman and Mescon[2] have recommended the following schedule for the staining of fungi.

Schedule for Staining of Fungi.—

1. Fix.
2. Dehydrate.
3. Deparaffinize sections and dehydrate through absolute alcohol.
4. Wash in distilled water.
5. Immerse in 1% periodic acid for 5 minutes.
6. Wash in running tap water for 15 minutes.
7. Stain in Schiff reagent for 10 to 15 minutes.
8. Transfer directly to two changes of the following solution for 5 minutes:

10% potassium metabisulphite	5 c.c.
1 N hydrochloric acid	5 c.c.
Distilled water	100 c.c.

9. Wash in running tap water for 10 minutes.
10. Counterstain with light green.
11. Dehydrate, clear, and mount.

Schiff Reagent.—

Dissolve 0.5 gm. of basic fuchsin by pouring over it 100 c.c. of boiling distilled water.

Cool to 50° C.

Filter and add 10 c.c. of 1 N hydrochloric acid and 0.5 gm. of anhydrous potassium metabisulphite to the filtrate.

Allow the solution to stand in the dark overnight. The solution should become colorless or pale straw color.

If not completely decolorized, add 0.25 to 0.50 gm. of charcoal, shake thoroughly, and filter immediately.

This solution will keep for several weeks in a tightly stoppered bottle.

The above schedule may be modified by the use of hematoxylin as a counterstain.

COLLECTION OF SPECIMENS

A few general principles governing collection of specimens will be emphasized by consideration at this point, although many of them will be repeated later in connection with discussion of specific mycoses. Except in the case of dermatophytosis, where a limited degree of drying is not detrimental and may, on the contrary, be beneficial by destroying some of the bacteria, it is important to insist upon fresh specimens. In the case of pulmonary disease, a freshly collected sputum specimen, preferably an early morning specimen, is essential. In a 24-hour specimen, growth of bacteria may occur in dense colonies, giving rise to a false diagnosis of actinomycosis. The bacterial growth may kill delicate fungi by antibiotic activity or at best interfere with the isolation of a fungus, although the addition of penicillin and streptomycin may overcome this handicap to a considerable extent. On the other hand, certain fungi such as *Candida albicans, Mucor,* and *Cephalosporium* grow readily and rapidly in sputum at room temperature, and the early appearance of many colonies on the culture slant will then lead to an erroneous estimation of their importance in the flora of the sputum and may obscure the primary etiology by overgrowing the actual pathogen. Prompt handling of other body fluids, pus, surgical material, and autopsy tissues is also desirable.

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[1] Lillie, R. D.: J. Lab. & Clin. Med. 32: 76, 1947.

[2] Kligman, A. M., and Mescon, H.: J. Bact. 60: 415, 1950.

PREPARATION OF CULTURES

Modified Sabouraud's agar (2 per cent glucose, 1 per cent neopeptone, 2 per cent agar, pH 6.5) is a satisfactory culture medium for most pathogens except *Actinomyces bovis.* For culture of sputum, ulcers, or draining pus, where bacterial contamination interferes with isolation of the fungus, penicillin and streptomycin should be added to the culture medium (after autoclaving) at a level of 40 to 60 units of each antibiotic per c.c. of agar. This culture medium must be stored in a refrigerator to retard inactivation of the antibiotics. The addition of Actidione (0.5 mg. per c.c.) to a medium is effective in retarding the growth of some saprophytic molds, while it permits the growth of such a pathogen as Coccidioides. Cultures may be incubated at room temperature, but incubation at 30° C. will enable earlier reading of the culture. Most of the fungi causing mycoses will grow at 37.5° C. in the laboratory, but primary isolation at this temperature is uncertain. Growth of most pathogens will be apparent after a few days but cultures should not be discarded as negative until they have been held at least one month.

EXAMINATION OF CULTURES

The experienced mycologist may be able to recognize a specific fungus by its characteristic appearance in a plate culture. In fact the rate of growth, surface configuration, texture, and color are major identifying characteristics among some species of dermatophytes. However, the primary basis for the identification of a mold is its microscopic morphology, particularly the size, shape, septation, and color of spores, and the manner in which spores are borne. It is essential, therefore, that a culture be examined when sporulation is most characteristic and the optimum time will vary, with the species, from 3 days to 3 weeks. It is generally useful to make the first examination when sporulation has just begun.

Drying and staining methods used in the study of bacteria are not useful.

The mycelium should be teased apart in a drop of mounting fluid and for this purpose two sharp, stiff, nichrome needles are essential. With one needle a bit of mycelium is picked up, touched to a drop of 95 per cent alcohol on the slide, and transferred immediately to an adjacent drop of 10 per cent sodium hydroxide.

With the two needles it is then gently spread in the drop.

The function of the alcohol is to wet the mycelium and eliminate air bubbles. Many mycologists prefer to use lactophenol and cotton blue as a mounting medium.

Lactophenol and Cotton Blue.—

Phenol crystals	20 gm.
Lactic acid	20 c.c.
Glycerol	40 c.c.
Distilled water	20 c.c.

Dissolve by heating over hot water. Add 0.5 gm. cotton blue.

ANIMAL INOCULATIONS

The inoculation of mice or guinea pigs for isolation of a fungus has a limited usefulness. It is effective in histoplasmosis, sporotrichosis, cryptococcosis, and coccidioidomycosis, and may be useful if the pathologic material is contaminated by saprophytic molds which are not inhibited by antibiotics added to the medium. Inoculation directly into the guinea pig testis sometimes produces an earlier infection than by other routes. In the case of histoplasmosis, the white mouse is a very susceptible animal and intraperitoneal injection of pus, ground tissue, or sputum is an effective method of isolating Histoplasma from contaminated material. Since sputum may contain pneumococci which cause early death of mice, penicillin (500 units per c.c. of sputum) should be mixed with the sputum before injection. Sacrifice the mouse 3 to 5 weeks after inoculation and make cultures from liver, spleen, omentum, and any abscesses or other lesions found.

The mouse is useful in the differentiation of virulent strains of *Cryptococcus neoformans* from morphologically similar nonpathogenic fungi. If 0.03 c.c. of a saline suspension containing 10,000 cells of a virulent strain is injected intracerebrally the mouse dies in 4 to 10 days and the encapsulated cells of *C. neoformans* are easily seen when a bit of brain tissue is crushed under a cover slip and examined microscopically. Mice similarly inoculated with avirulent strains or with yeasts survive, and cultures made from the brain after one month are usually sterile.

DERMATOPHYTOSIS

(Ringworm, Tinea, Athlete's Foot)

Dermatophytosis is caused by the invasion of the skin, hair, or nails by one of several closely related keratolytic fungi known collectively as dermatophytes. Lesions may be characterized by barely perceptible scaling of glabrous skin or palmar surfaces, extensive scaling with infiltration at the advancing border, vesiculation, or dense boggy areas, depending upon the species of fungus involved and the reaction of the host. Lesions involving the scalp may present alopecia, areas showing many broken hair stubs, or infiltrated areas (kerion). Infections of the nails (onychomycosis) may be characterized by thickened nails or chalky degeneration of nail tissue sometimes resulting in almost complete destruction of the nail.

In all types of dermatophytosis microscopic confirmation of the presence of a fungus and its isolation in culture are essential in the differential diagnosis. Clinical differentiation from dermatitis is often impossible.

Collection of Material

In lesions of the skin, thorough cleansing of the lesions with 70 per cent alcohol is desirable. This removes much of the superficial dead tissue in which the fungus cannot be demonstrated readily, it eliminates many contaminating bacteria and saprophytic mold spores, and it may remove ointments which make microscopic examination difficult. Specimens should be taken by stripping back the epidermis at the advancing edge of the lesion with forceps. If vesicles are present the roof of a vesicle may be clipped off with scissors, although many vesicular lesions represent an allergic reaction and are sterile. Nails should be scraped deeply to obtain freshly invaded tissue. In lesions of the scalp broken hair stubs should be collected. The selection of infected hairs can be facilitated by the use of a filtered ultraviolet lamp (Wood light) under which infected hairs fluoresce. Epidermal scales, nails, and ointments also fluoresce with varying degrees of brilliance and may confuse the inexperienced.

Specimens taken as suggested above are suitable for either direct microscopic examination or culture.

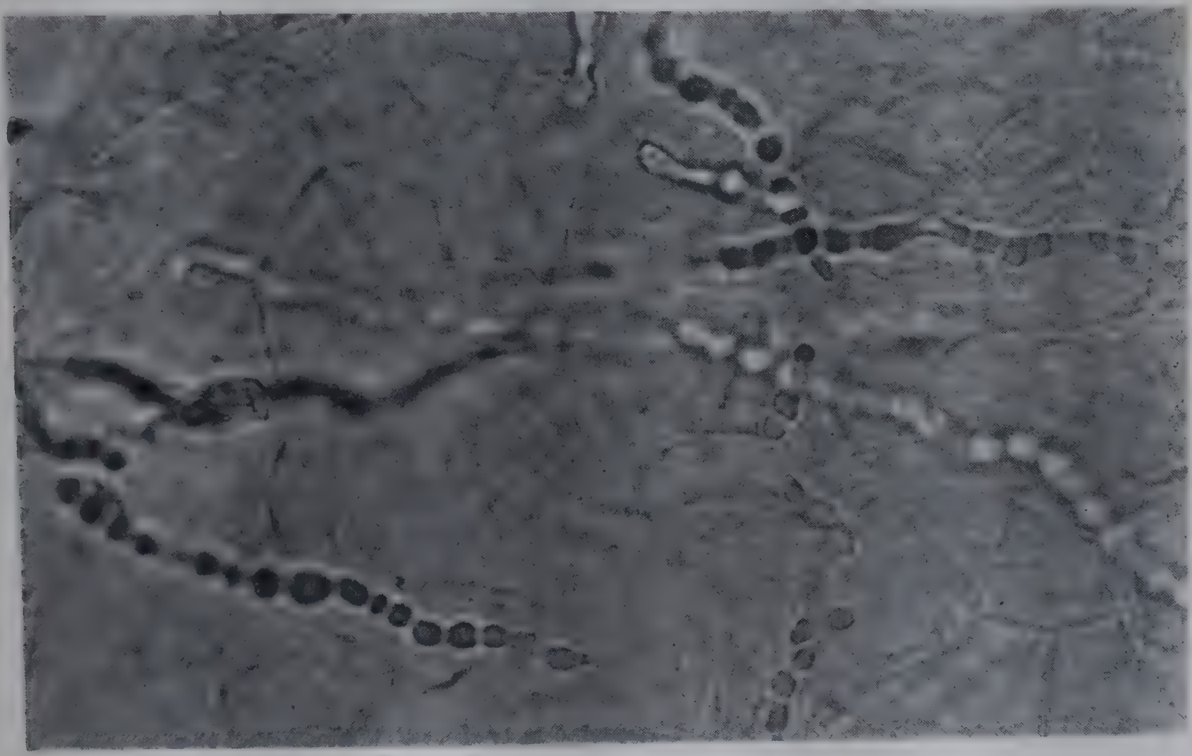

Fig. 412.—Hyphae of Trichophyton in epidermis. (×500.)

Microscopic Examination

Place the specimen (small pieces of epidermal scales, finely divided nail or hairs) on a microscope slide in a drop of 10 per cent sodium hydroxide, put a cover glass in place, heat the slide for a few seconds over a low flame until the specimen softens (but without boiling), and then examine under the microscope.

In epidermal scales and nails the fungus is seen as branching hyphae running through and between epidermal cells (Fig. 412). It is necessary to differentiate borders of epidermal cells, "mosaic fungus," and fibers. The "mosaic fungus" (Fig. 413) is an artefact consisting of cholesterol deposited in a mosaic pattern between epidermal cells.

Cotton fibers can be differentiated from dermatophyte hyphae by their flatness and varying width, wool fibers by their pattern of overlapping cells, and nylon and silk by the absence of cross walls and branching.

In hairs the hair shaft is usually invaded and, depending upon the species of fungus, there may or may not be a sheath of arthrospores outside the hair shaft.

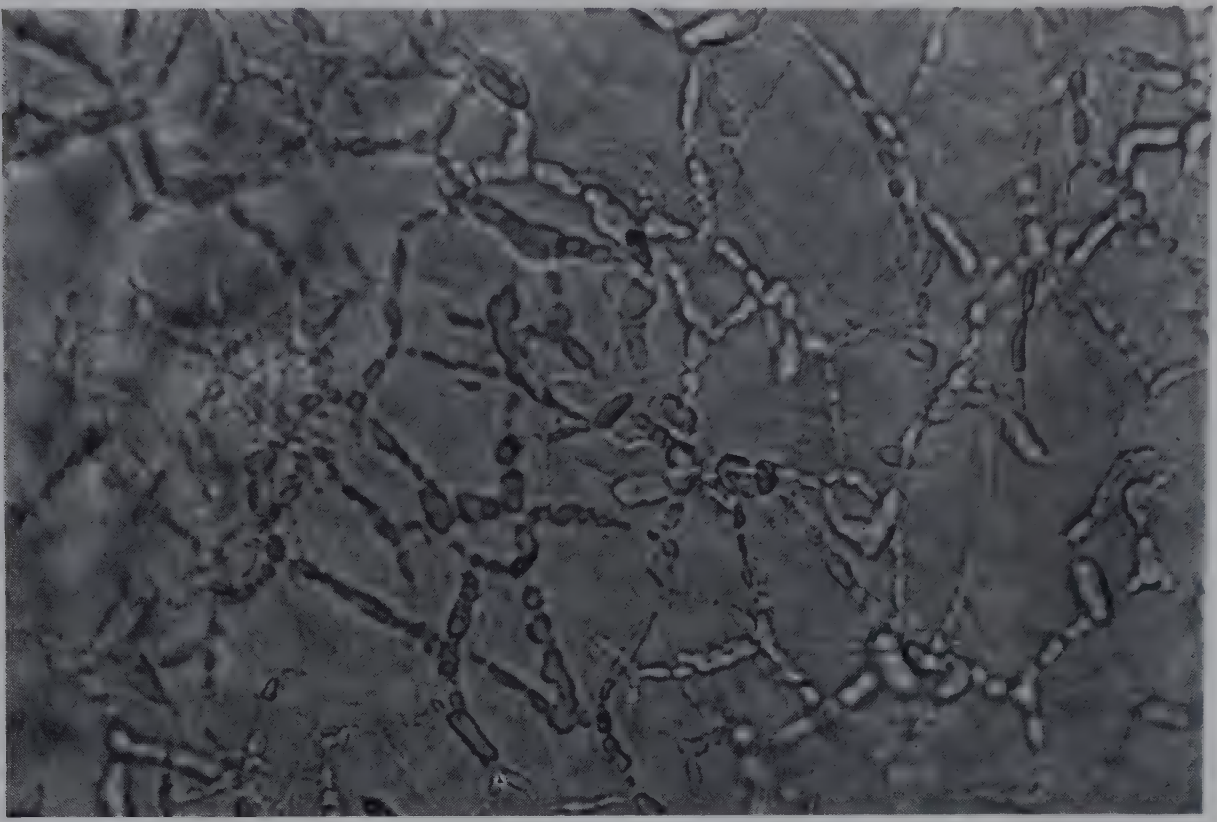

Fig. 413.—"Mosaic" consisting of cholesterol, an artefact to be differentiated from fungus hyphae. (×500.)

Cultures

Specimens may be planted on Sabouraud's agar, fortified with penicillin and streptomycin as indicated above. If it is inconvenient to use antibiotics in the medium, contaminating bacteria can be effectively eliminated from small epidermal and nail specimens by immersion in 70 per cent alcohol in a Petri dish. The bactericidal action of 70 per cent alcohol is greater than its fungicidal action. Begin transferring specimens to agar slants after 2 minutes' exposure to the alcohol, and take the final specimens before 8 minutes have elapsed. Cultures should be incubated at 30° C. or room temperature. Many dermatophytes grow promptly and can be identified in 7 to 10 days, but cultures should be held for one month before being discarded as negative.

Classification of Dermatophytes

Medical literature contains several hundred binomials for the dermatophytes. Most of these names are the result of taxonomic revisions or the creation of a new specific name on the basis of a slight variation in some characteristic. Modern usage based on mycologic criteria has reduced the number of genera to three: Trichophyton, Microsporum, and Epidermophyton.[1] The salient features of these three genera are illustrated in Fig. 414. There are perhaps 15 or 20 valid species.

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[1] Emmons, C. W.: Arch. Dermat. & Syph. 30: 337, 1934.

TRICHOPHYTON Malmsten 1843

Trichophyton is the largest of the three genera in number of species and in frequency with which it attacks man. In lesions of the epidermis, Trichophyton produces branching hyphae which break up into arthrospores by septation and there are no characteristics which differentiate it from the other two genera. In the scalp, some species of Trichophyton which attack the hair produce also a sheath of spores surrounding the hair shaft. These spores are arranged in a linear pattern easily differentiated from the mosaic pattern seen in Microsporum infection (Fig. 415).

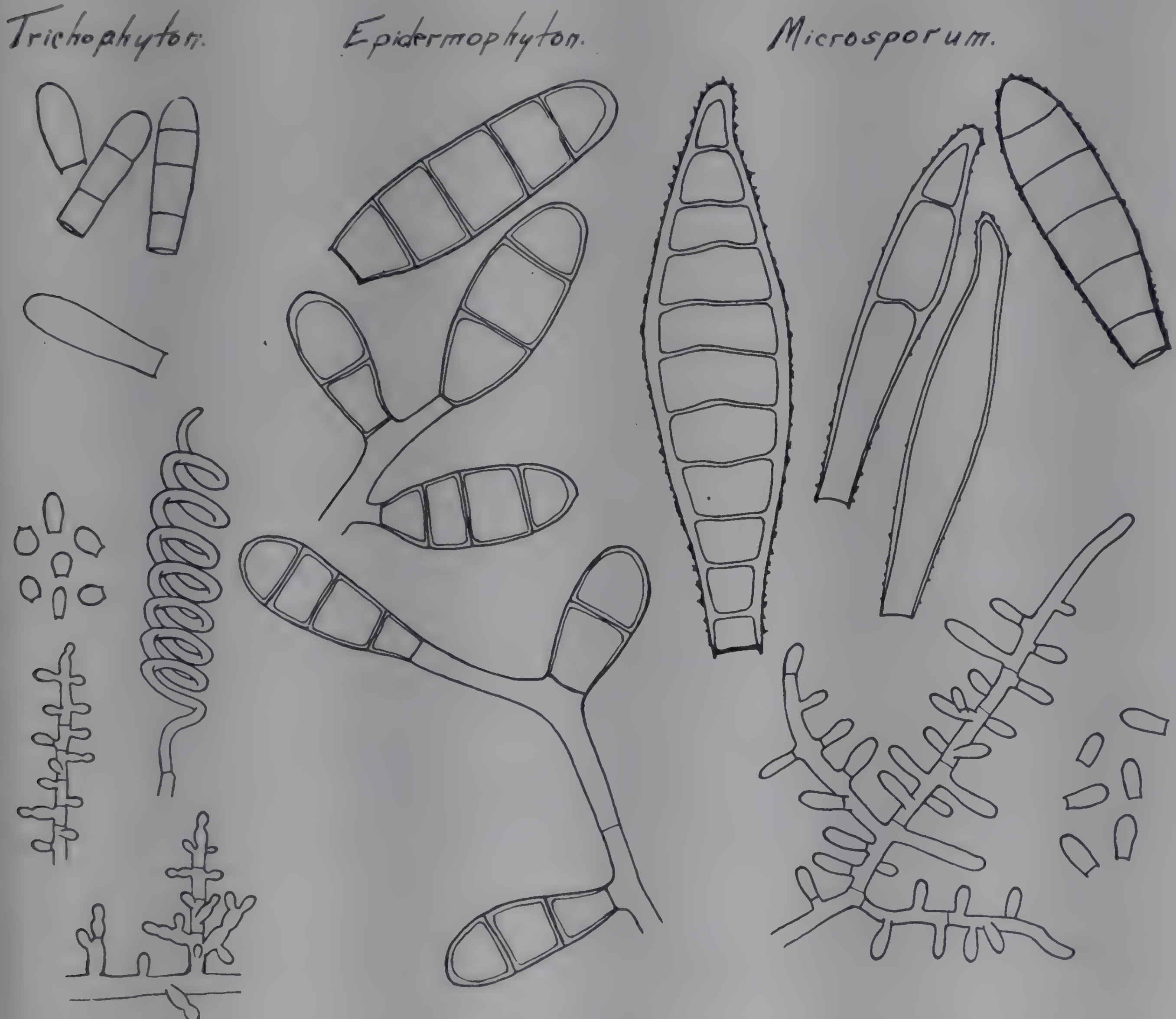

Fig. 414.—Differential characteristics of the three genera of dermatophytes. The macroconidia of Microsporum represent, from left to right, *M. canis*, *M. audouini*, and *M. gypseum*. (×450.) (Adapted from Emmons, C. W.: Arch. Dermat. & Syph. 30: 337, 1934.)

Trichophyton is characterized by conidia which average 2 by 2 to 4 microns, are single-celled, hyaline, thin-walled, subspherical to clavate (club-shaped), and have a broad base at the point of attachment to the conidiophore, and by macroconidia which are 4 to 6 by 10 to 50 microns, with a blunt distal end, one to several-celled, with smooth, relatively thin walls. The shape and septation of the macroconidia differentiate Trichophyton from Microsporum and Epidermophyton. The conidia are borne laterally on the hyphae or on simple or branched conidiophores (Figs. 414, 416).

Trichophyton mentagrophytes (Robin) Blanchard, 1896 *(T. gypseum, T. interdigitale)*, is the commonest species of the genus. It includes several varieties which produce colonies varying in texture from chalky to floccose (Fig. 417). The color of a colony varies usually from white to cream, but some strains develop pink to brownish shades. It is one of the etiological agents of athlete's foot, onychomycosis, kerion, and tinea circinata of the type acquired from animals.

Trichophyton rubrum (Castellani) Sabouraud, 1911, is characterized by narrow, clavate macroconidia and production of a red pigment. It is associated with athlete's foot, onychomycosis, and tinea cruris and has a tendency to become generalized over the body, particularly in skin folds and about the waist. Although it appeared at one time to be largely subtropical in distribution, it now commonly seen over the United States.

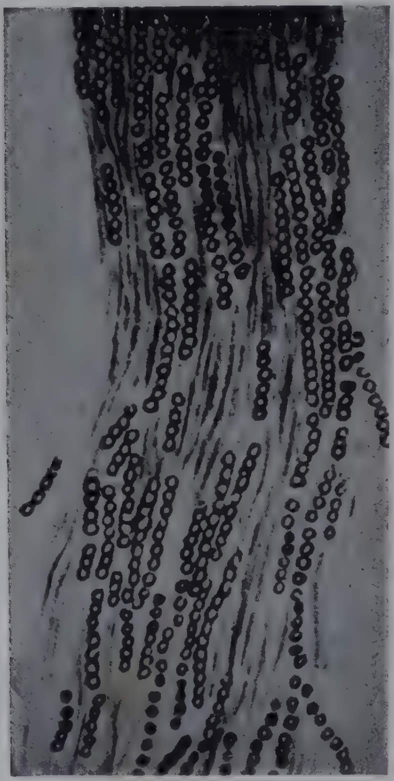

Fig. 415.

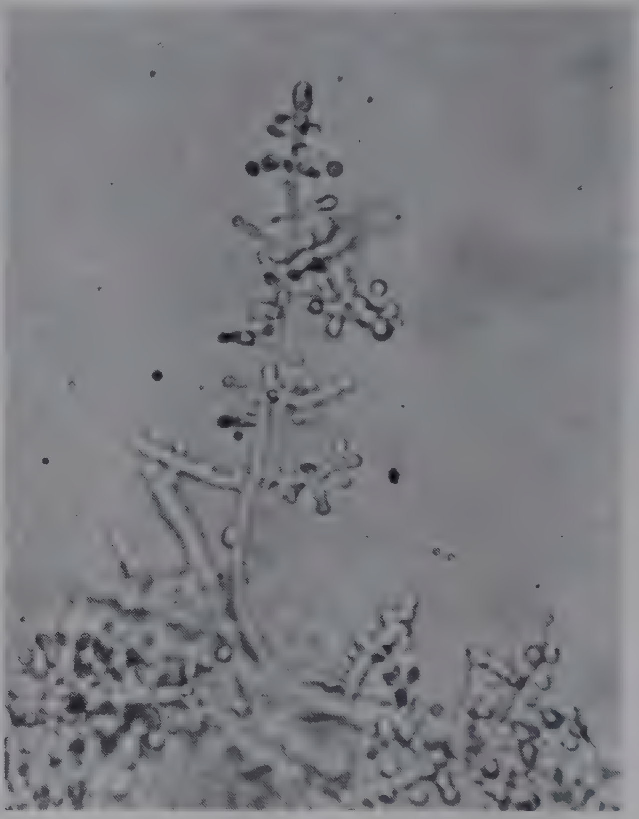

Fig. 416.

Fig. 415.—*Trichophyton gypseum asteroides* in hair. (From Bruhns-Alexander: Grundriss der mykologischen Diagnostik, Julius Springer, Berlin.)

Fig. 416.—Conidiophore and conidia of *Trichophyton mentagrophytes*. (×250.)

Trichophyton tonsurans Malmsten, 1845, is representative of a group of species associated primarily with ringworm of the scalp. Growth is slow, colonies develop short aerial mycelia, and the surface of the colony is folded, often in a characteristic concentric, radial, or cerebriform pattern. Conidia show somewhat more variation in size than the preceding species but are otherwise similar, and macroconidia are rare.

Trichophyton schönleini (Lebert) Langeron and Miloch., 1930, causes favus. Growth is slow, producing glabrous, wrinkled, and folded colonies with relatively poor production of spores. *Trichophyton verrucosum* Bodin, 1902 *(T. faviforme)*, resembles *T. schönleini* in culture characteristics, and represents the "faviform group" of dermatophytes which are primarily pathogens of cattle but are seen in men having contact with cattle. For a fuller description of these and other species the reader is referred to Conant and associates.*

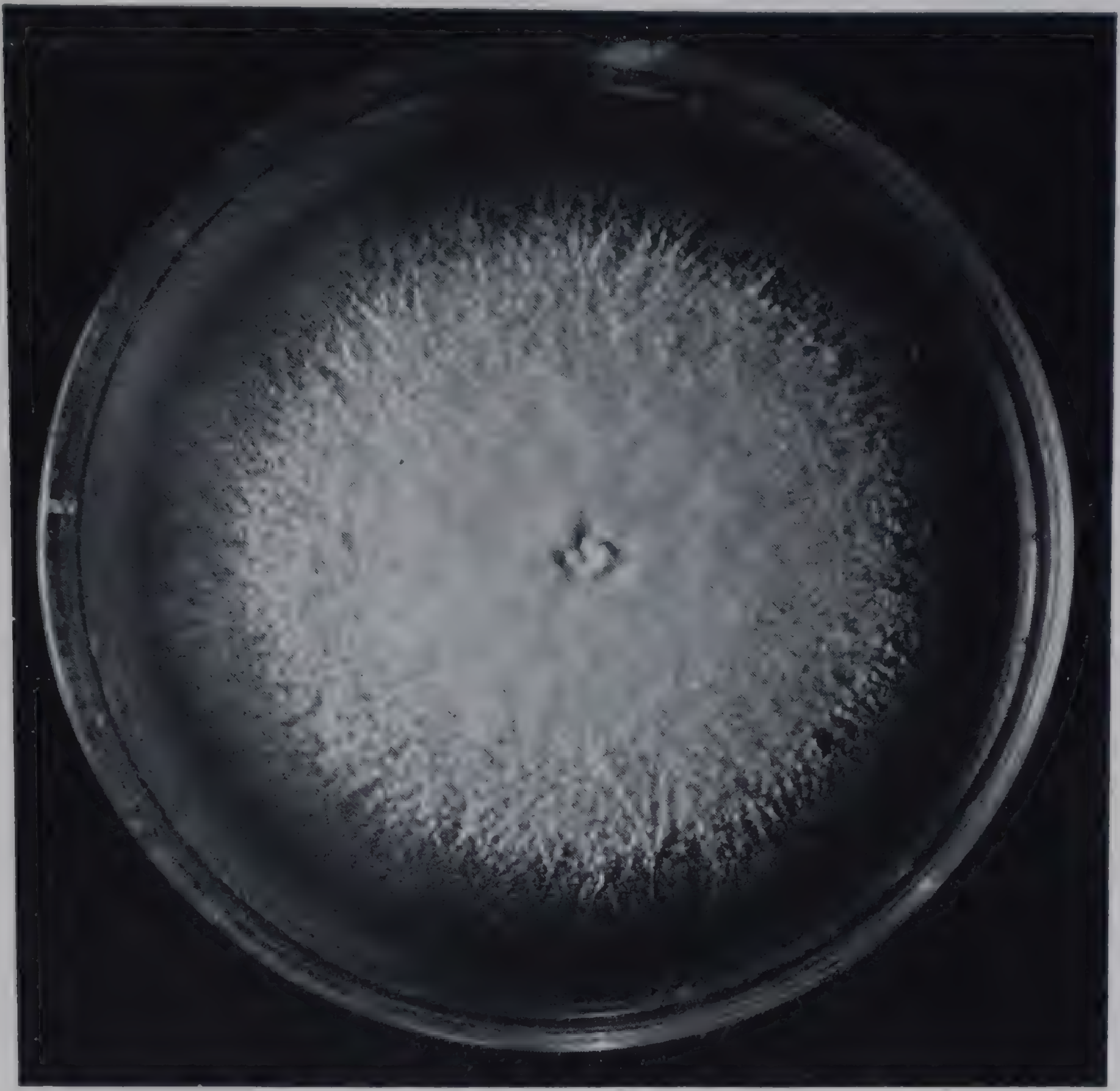

Fig. 417.—Colony of *Trichophyton mentagrophytes* after 2 weeks' incubation at 30°C. on Sabouraud's agar.

MICROSPORUM Gruby 1843

Microsporum causes ringworm of the scalp and occasionally circinate lesions of the glabrous skin. In lesions of the epidermis Microsporum cannot be differentiated from Trichophyton, but in lesions of the scalp there is a characteristic appearance in the hair. The hair shaft is invaded, as in Trichophyton, but the spores on the surface of the hair are arranged in a mosaic pattern easily differentiated from the linear arrangement of Trichophyton arthrospores (Fig. 418).

The genus is characterized in culture by conidia which are somewhat larger and more generally clavate than those of Trichophyton (2 to 3 by 4 to 6 microns) and more particularly by large spindle-shaped macroconidia 8 to 16 by 35 to 160 microns in size (Fig. 414). The walls of the macroconidia are thick and marked with spines or warts on the external surfaces, especially toward the narrow, pointed ends.

Microsporum canis Bodin, 1902 *(M. felineum, M. lanosum)*, is a zoophilic species which commonly causes ringworm in cats and dogs and these hosts are the usual sources of infection in the sporadic human cases of ringworm caused by this species. It grows rapidly, producing a floccose colony with a characteristic yellow color, and it bears large numbers of macroconidia. These have 8 to 15 cross walls which are sometimes diagonally oriented (Fig. 414). A varietal form differing in absence of the typical yellow color is sometimes isolated.

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*Loc. cit., p. 1764.

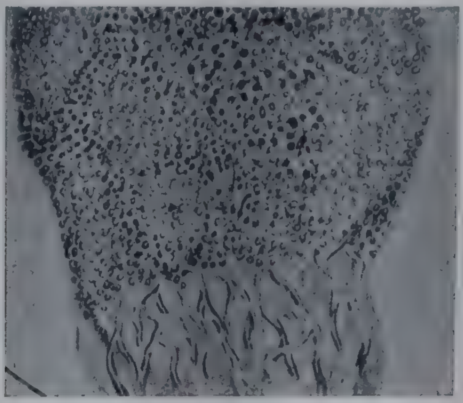

Fig. 418.—Hair infected with *Microsporum audouini.* (From Bruhns-Alexander: Grundriss Grundriss der mykologischen Diagnostik, Julius Spring, Berlin.)

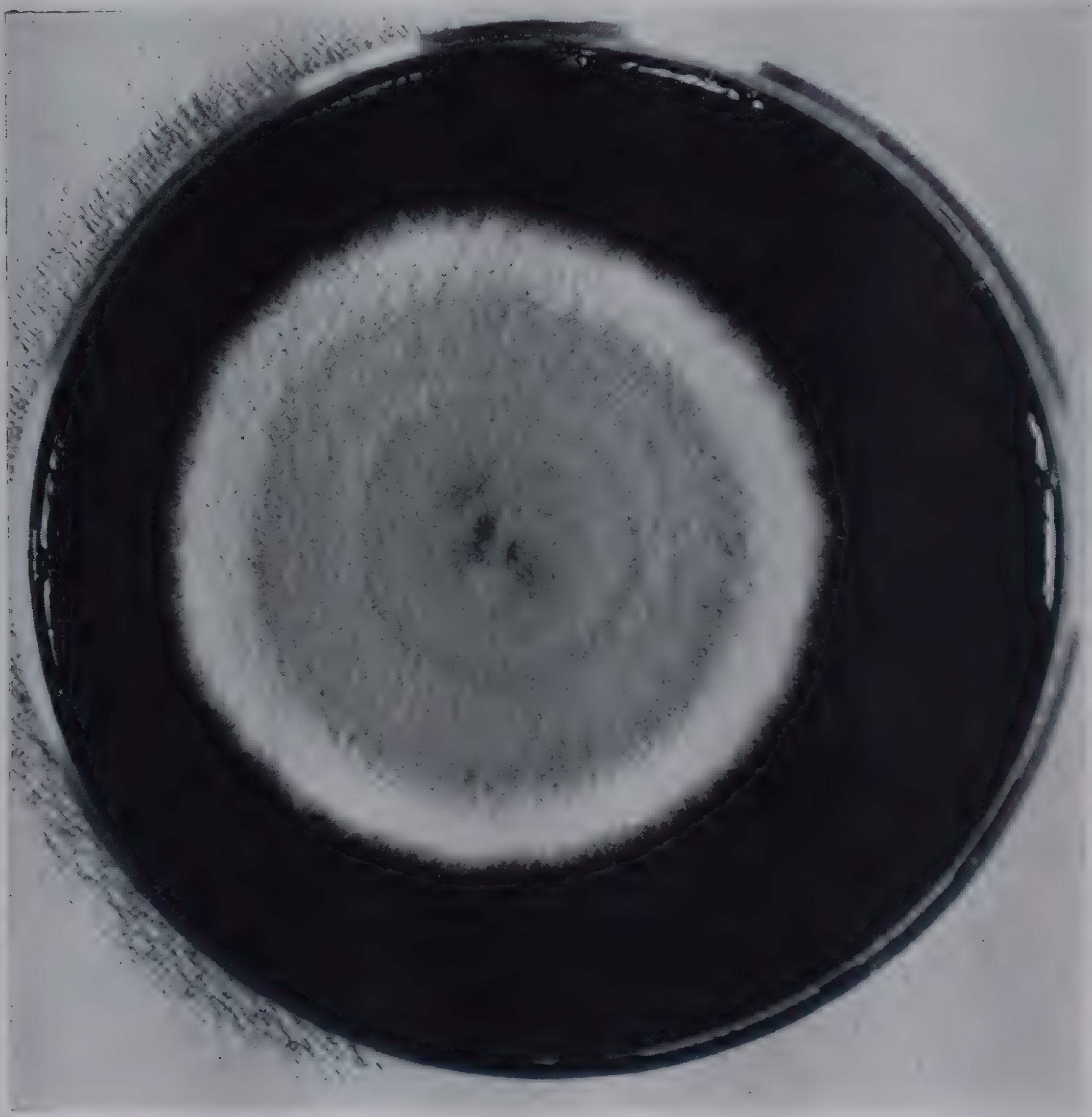

Fig. 419.—Colony of *Microsporum gypseum* after 2 weeks' incubation at 30°C. on Sabouraud's agar.

Microsporum audouini Gruby, 1843, is the cause of epidemics of ringworm of the scalp in children. Spontaneous recovery occurs at the age of puberty and the species is rarely isolated from adults. The fungus grows rather slowly and produces a gray to brownish, velvety colony and a reddish brown pigment in the reverse side of the colony. Sporulation is sparse and macroconidia are rare and usually small, bizarre in shape and with few septa (Fig. 414).

Microsporum gypseum (Bodin) Guiart and Grigorakis, 1928, grows rapidly and produces a fawn or buff to brown colony in which macroconidia are abundant (Fig. 419). These macroconidia are smaller than those of *M. canis,* have commonly 6 to 8 septa, and have rounded rather than pointed ends.

EPIDERMOPHYTON Sabouraud 1910

The genus contains a single species, *Epidermophyton floccosum* (Harz) Langeron and Miloch., 1930, which causes athlete's foot and tinea cruris. It does not invade the hair shaft. In culture, *E. floccosum* produces a greenish yellow flat colony in which only macroconidia are produced. The macroconidia are borne as sessile or short stipitate lateral spores on vegetative hyphae or in groups on simply branched conidiophores. They are egg-shaped to clavate, with thick, smooth walls and may be nonseptate or have 1 to 4 cross walls (Fig. 414).

CANDIDIASIS

(Moniliasis)

Candida albicans (Robin) Berkhout, 1923, is a yeastlike fungus found frequently on mucous membranes where it causes thrush in infants and vaginitis, especially during pregnancy. It is a secondary invader in most types of pulmonary disease, where its etiological significance may be difficult to evaluate. It also causes paronychia and epidermal erosions in skin folds. A small number of cases of endocarditis caused by *C. parapsilosis* (and rarely by *C. albicans*) have been reported.

The taking of specimens from the oral mucosa, skin, and blood presents no special problems. In the examination and culture of sputum it is important to collect a fresh morning specimen, for reasons given above. Material may be examined directly in 10 per cent sodium hydroxide or in a Gram-stained smear and planted on glucose-neopeptone agar with added penicillin and streptomycin.

Candida is of sufficient size (2.5 to 3.5 by 3 to 5 microns) so that its characteristic oval or elliptical cell, often with a bud attached at one end, or its hyphal form, may be found under the microscope without staining. However, sputum may be stained by the Gram method. There is some variation in staining reaction depending upon the age of the cell, but most young cells are strongly gram-positive. Candida is easily isolated in culture from sputum or body fluids or surfaces even in the presence of bacteria which might inhibit other pathogens. On glucose-neopeptone agar, Candida grows first as a yeast, forming a glistening, creamy white, heaped colony, the surface of which is often pitted with the scars of gas bubbles. The important pathogenic species is easily recognized by planting on corn-meal agar or the special medium of Nickerson and Mankowski.[1] Inoculate with a deep streak on a plate or slide culture and incubate at room temperature. *C. albicans* produces spherical chlamydospores which are distinctive (Fig. 420). *C. albicans* is further identified by production of acid and gas in glucose and maltose and of acid in sucrose and by growth in glucose broth without clouding or formation of a surface film.[1]

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[1] Nickerson, W. J., and Mankowski, Z.: J. Infect. Dis. 92: 20, 1953.

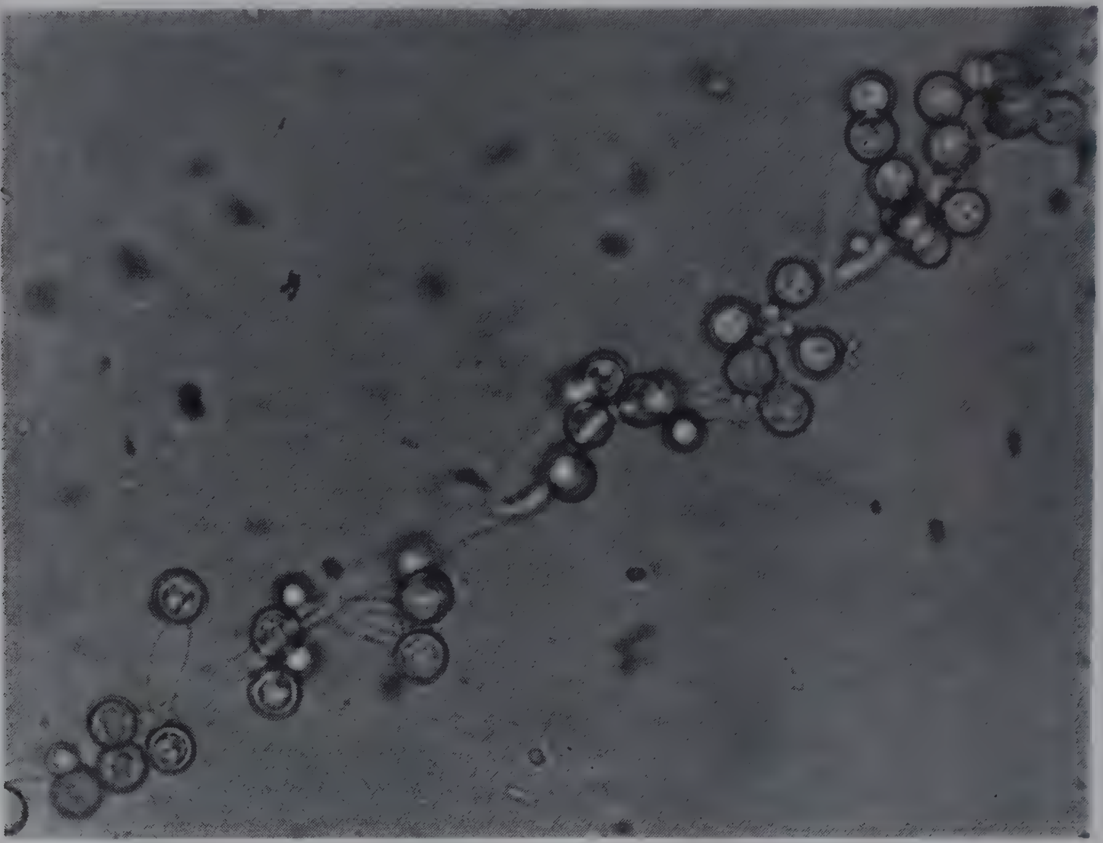

Fig. 420.—Chlamydospores of *Candida albicans*. (×500.)

ACTINOMYCOSIS

Actinomycosis is a systemic, chronic, granulomatous and suppurative disease which involves the face and neck in a little more than half the cases, the thoracic organs in about a fourth, and the abdominal organs, skin, or meninges in the remainder.

Small colonies of the fungus, which are often in the form of well-organized "sulphur granules" with peripheral "clubs" of eosinophilic material deposited around the hyphal tips, are characteristically found in the pus which discharges from sinus tracts leading from the lesion (Fig. 421). These granules can be seen with the unaided eye in pus as it flows down the side of a glass tube or on the surface of gauze dressings. Unless pus from unopened sinuses can be aspirated the granules from a draining sinus should be collected for examination and culture.

When crushed and stained by the Gram method, fragile gram-positive hyphae 0.5 to 1.0 micron in diameter, which branch but break up readily into diphtheroid elements, are seen (Fig. 422). For purposes of culture the granules should be washed rapidly in sterile saline and planted in semisolid glucose thioglycollate medium or on the surface of blood agar or other enriched medium in an anaerobic jar. Growth occurs at 30° to 37° C.

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[1] Conant and others: Loc. cit

Actinomyces bovis Harz, 1877, the cause of actinomycosis, is microaerophilic. In culture its microscopic morphology is much like that seen in tissues. A branching mycelium can be demonstrated in a culture two or three days old, but in most strains the hyphae break up in older cultures into diphtheroid elements (Fig. 423). *A. bovis* must be differentiated from microaerophilic organisms having similar morphology which frequently appear in culture and which probably are saprophytic contaminants of the skin. These saprophytes grow more rapidly than *A. bovis* and produce a profuse growth

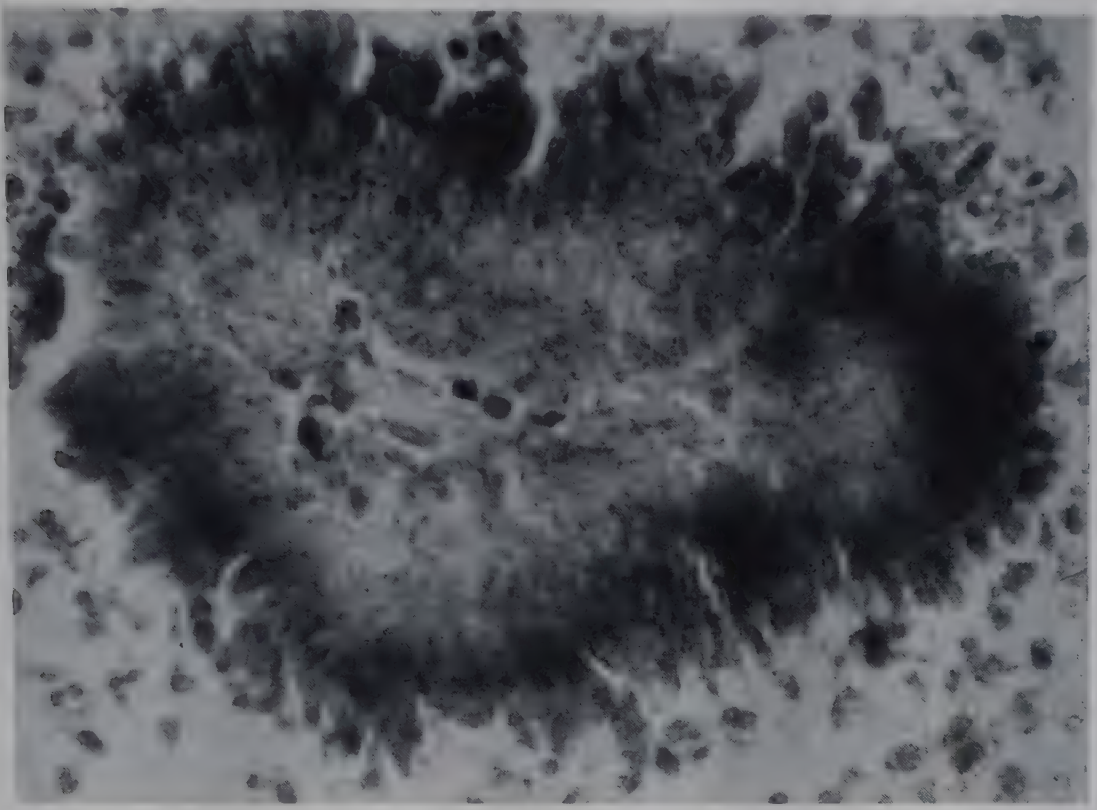

Fig. 421.—Section of a "sulphur granule" from actinomycosis. (×500.) (From Emmons, C. W.: Mycologia 24: 377, 1937.)

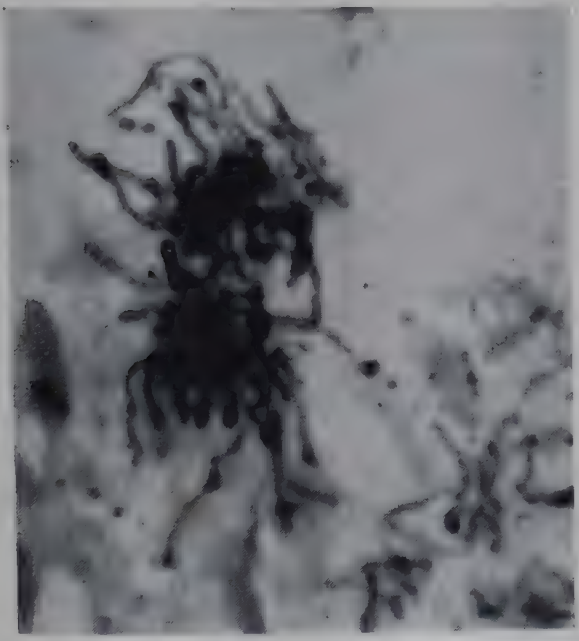

Fig. 422.

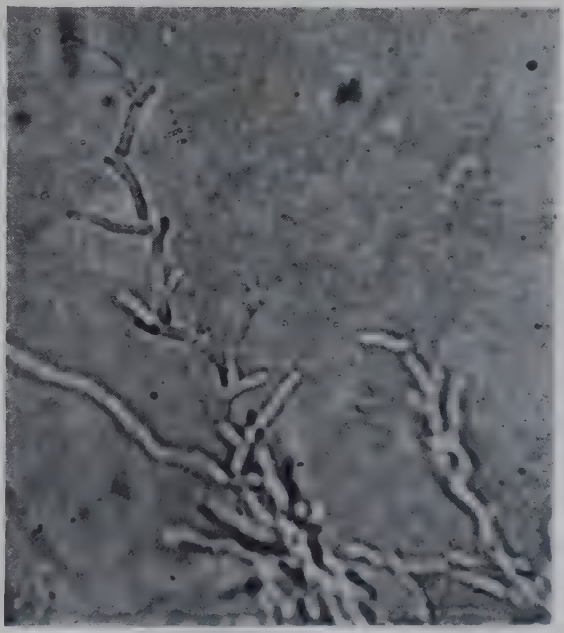

Fig. 423.

Fig. 422.—Gram stain of *Actinomyces bovis* from a crushed granule. (×900.)

Fig. 423.—The fragile branching mycelium of *Actinomyces bovis* from a young culture. (×900.) (From Emmons, C. W.: Puerto Rico J. Pub. Health Trop. Med. 11: 63, 1935.)

which settles to the bottom of a semisolid medium. Cultures remain viable after several weeks' incubation. *A. bovis,* on the other hand, remains in discrete colonies and must be transferred at 10-day intervals under ordinary conditions to maintain a strain. *A. bovis* is further differentiated by its ability to produce abscesses in young hamsters after intraperitoneal inoculation, while the saprophyte is not pathogenic.

MYCETOMA

A mycetoma is a granulomatous lesion in the subcutaneous tissues, usually of an extremity, characterized by swelling, the formation of sinus tracts, and the discharge of pus which contains granules composed of dense fungus colonies. The foot is most commonly involved (Madura foot) and the swelling results in a characteristic convex contour of the sole. Infection follows penetration of the skin by a splinter or thorn contaminated by the fungus.

One should speak of ''mycetomas'' since the clinical term covers infections of diverse etiologies. The fungi range from species of the Nocardia, which are of bacterial dimensions, to coarse Hyphomycetes such as Monosporium. Some of the fungi causing mycetoma have been isolated from soil and such a saprophytic occurrence of the others is assumed.

Nocardia madurae (Vincent) Blanchard, 1896, produces yellowish white granules in the tissues. These granules are firmer than those of actinomycosis and, when crushed, the component hyphae are not so easily dissociated. The hyphae are approximately 1 micron in diameter and are gram-positive and not acid-fast. In culture, *N. madurae* is aerobic and produces grayish-white to yellow, spreading waxy, glabrous colonies. Under some conditions of growth, colonies may be pink to red. *N. madurae* was first isolated in India, whence came the name, but it occurs also in the United States and elsewhere in the world.

Nocardia brasiliensis (Lindenberg) Castellani and Chalmers, 1913, also produces yellowish-white granules in tissue, but in culture produces a yellow to orange, heaped or cerebriform colony which may develop a chalky surface due to formation of short aerial hyphae. *N. brasiliensis* causes mycetoma in southern United States, Mexico, and Central and South America.

Nocardia pelletieri (Laveran) Pinoy, 1912, is found in tissues in the form of red granules, and in culture produces a coral to ruby red colony. The present criteria for identification of species of Nocardia and their separation from Streptomyces are inadequate but are currently under study.

Besides Nocardia, some 20 of the higher fungi cause mycetoma. Allescheria, Aspergillus, Penicillium, Madurella, Indiella, Glenospora, Cephalosporium, and Phialophora are each represented by one or more species. Ten species of Madurella producing black grains have been described, but it is probable that some of these should be reduced to synonymy.

Mycetomas caused by the higher fungi can be recognized by the greater diameter of the hyphae making up the ''grain.'' These are 2 to 4 microns in diameter although there are occasional larger intercalary cells, and many of the radiating hyphae are terminated at the periphery of the granule by cells reaching a diameter of 6 to 8 microns.

In the United States, *Allescheria boydii* Shear, 1921, which causes a white grain mycetoma, is the most important representative of this group. For more than 20 years only the single original case reported in 1921 was attributed to this fungus. However, during this period numerous cases of mycetoma caused by *Monosporium apiospermum* Saccardo, 1911, were reported. Comparisons of these two fungi showed that they are actually one. *A. boydii* is an Ascomycete and exhibits the full complement of spore forms known for the fungus. *M. apiospermum* (a member of the Class Fungi Imperfecti) is the name applied to those strains of the fungus usually isolated from mycetoma which lack the ascomycetous stage and reproduce by conidia only. *A. boydii* has now been isolated frequently from soil and it is interesting to note that these strains of saprophytic origin exhibit the full life cycle of the fungus.

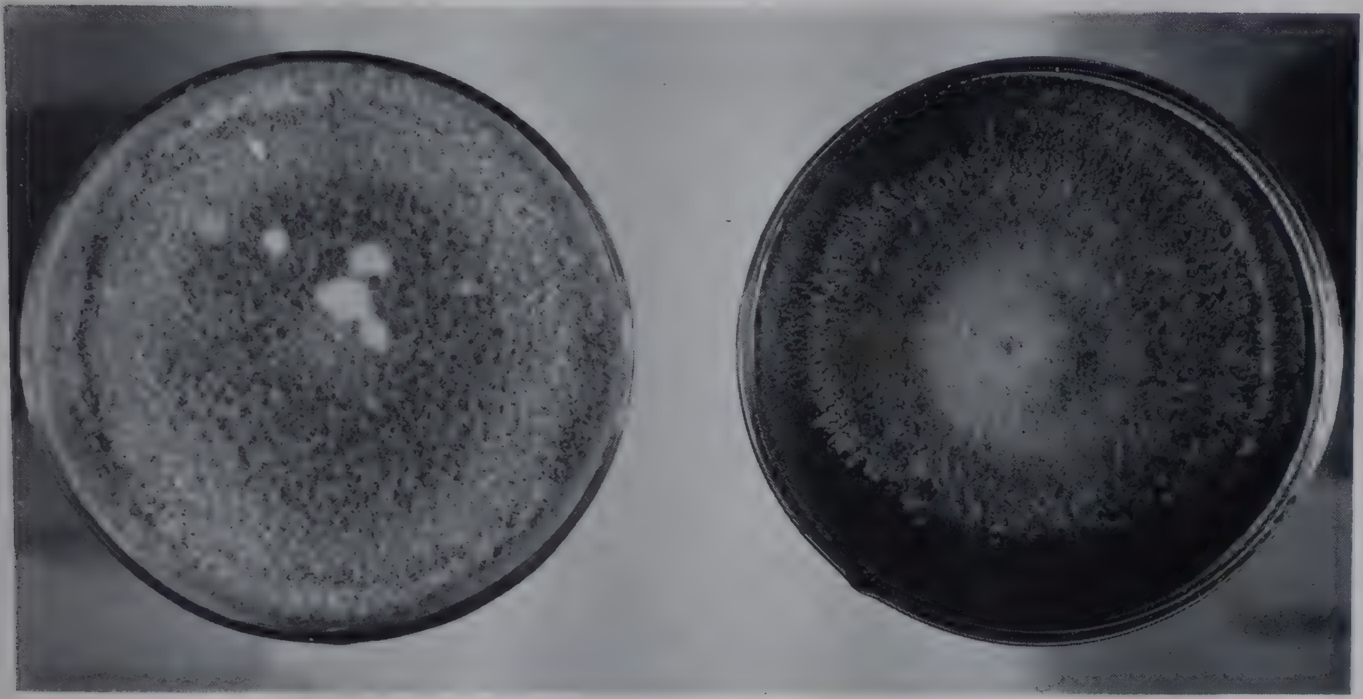

Fig. 424.—*Allescheria boydii* cultures 4 weeks old. Sabouraud's agar at left, corn-meal agar at right.

Grains from an unopened abscess should be planted upon Sabouraud's agar. If grains from a draining sinus are used they should be washed briefly in sterile saline and planted on a medium containing antibiotics. Cultures should be incubated at 30° C. The fungus grows rapidly, spreading widely over the slant, and producing a floccose gray to brownish-gray colony (Fig. 424).

Sporulation can be studied most conveniently upon corn-meal agar which reduces the amount of vegetative growth and stimulates sporulation. Two types of spores are produced. Conidia are one-celled, subspherical to short clavate, 5 to 7 by 8 to 11 microns; the base presents a broad surface where it was attached to the conidiophore (Fig. 425). They are borne terminally and singly at the ends of hyphae or short side branches. The conidiophores are sometimes associated into bundles called "coremia." The ascospore is elliptical and resembles the conidium sufficiently in size and shape so that it has been frequently overlooked in superficial examinations. Its slightly smaller size, 4 to 5 by 5.5 to 7.5 microns, and elliptical shape with no broad basal scar at the point of attachment serve to identify it. Ascospores are borne 8 per ascus within a spherical structure called an "ascocarp." The ascocarp wall (the perithecium) is composed of a single layer of specialized flattened cells. This perithecial wall bursts at maturity to liberate the ascospores (Fig. 426).

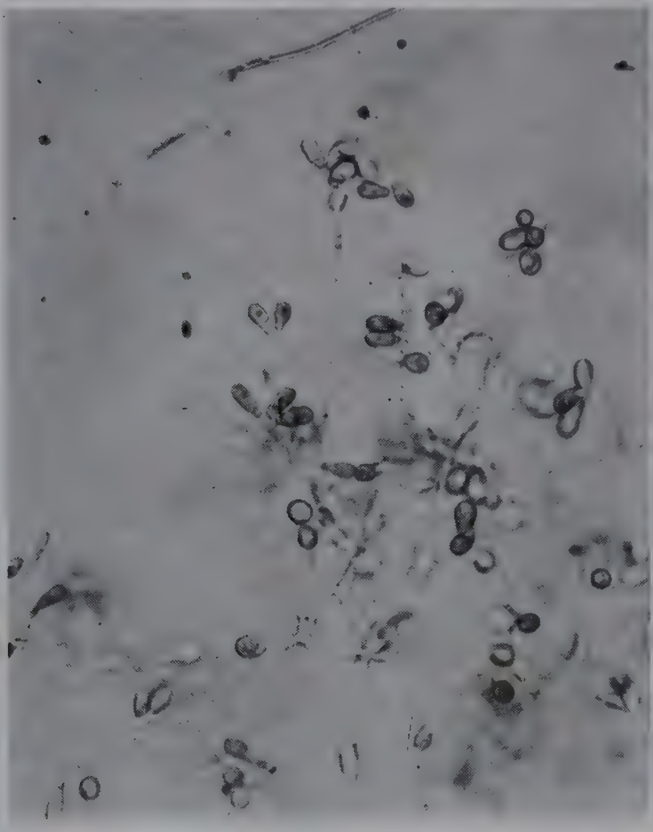

Fig. 425.

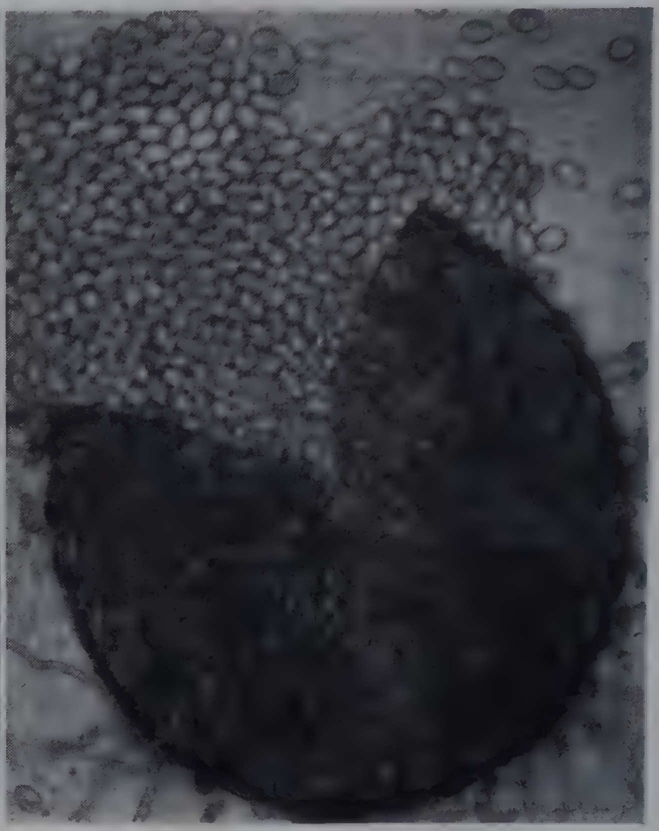

Fig. 426.

Fig. 425.—*Allescheria boydii* conidia. (×250.) (From Emmons: Mycología 36: 188, 1944.)

Fig. 426.—*Allescheria boydii* mature ascocarp and ascospores. (×250.) (From Emmons: Mycología 36: 188, 1944.)

CHROMOBLASTOMYCOSIS

Chromoblastomycosis is a chronic infection of the skin and subcutaneous tissues characterized by warty sessile or pedunculated lesions and by swelling due to lymph stasis. It is seen most frequently in tropical and subtropical climates but this may be due to more frequent exposure to the traumatic introduction of the fungus via contaminated thorns.

Microscopic examination of superficial crusts and epidermal scales cleared in 10 per cent sodium hydroxide reveals brown fungus hyphae and sclerotic cells in the epidermis. Sections of a lesion show hyperkeratosis and cutaneous abscesses in which the fungus is present as brown, thick-walled subspherical cells, 5 to 12 microns in size, which reproduce by septation rather than budding. Cultures may be made by planting crusts, aspirated pus, or ground tissue from a biopsy on Sabouraud's agar fortified by antibiotics and incubated at 30° C.

A mycologic discussion of chromoblastomycosis is complicated by the fact that any one of three fungi may cause the same clinical entity and by the diversity of sporulation which these obviously related fungi exhibit.[1] The diversity is so great that the three fungi have been placed at different times and by various investigators in several different genera, yet they have characteristics in common which have led other mycologists to place them within a single species. In my opinion the three are closely related genetically and belong in one genus, but it is convenient at present to assign them to three species.

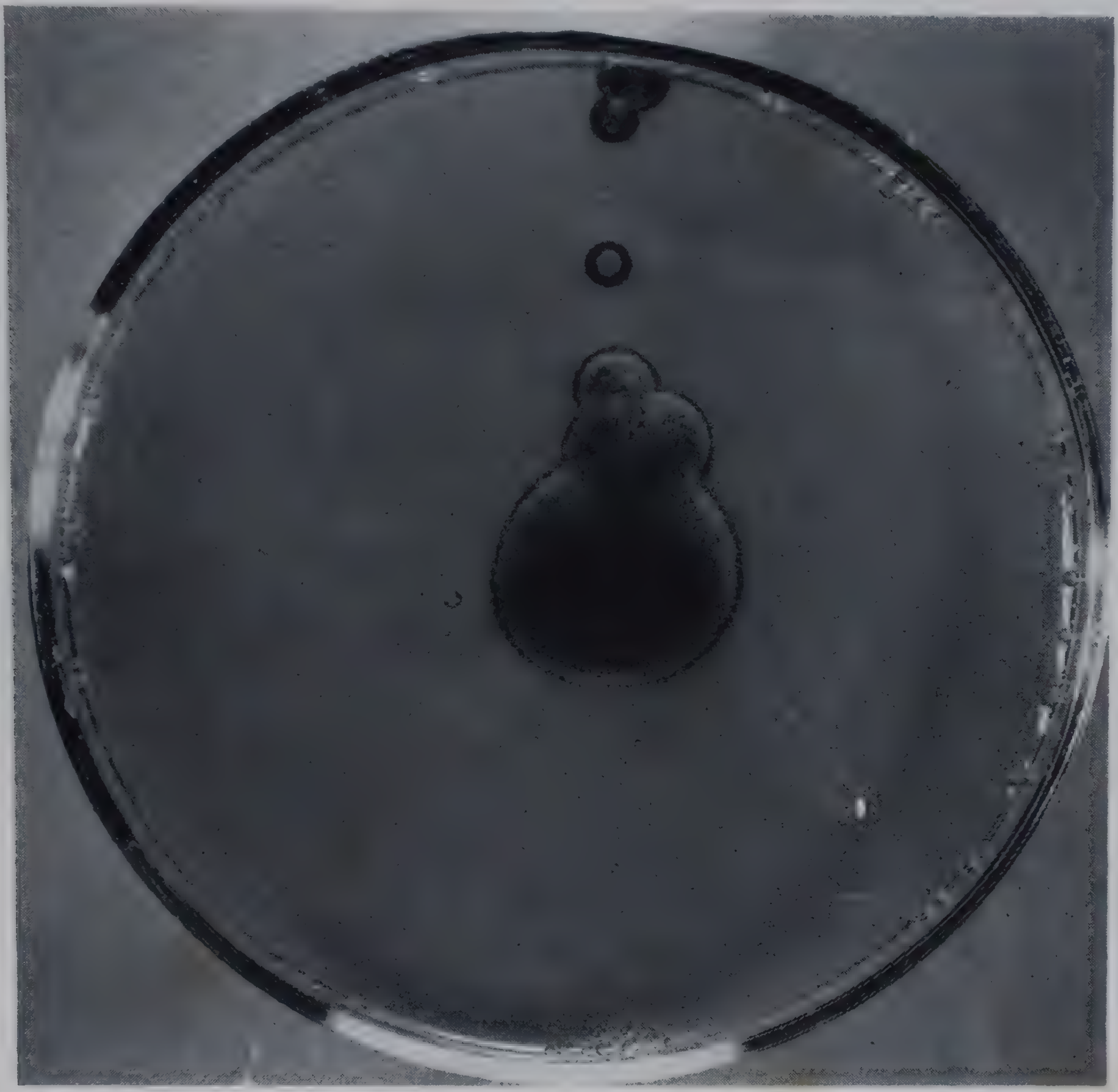

Fig. 427.—Colony of *Phialophora verrucosa* incubated 2 weeks at 30°C on Sabouraud's agar.

Phialophora verrucosa Thaxter, 1915 *(Cadophora americana, Fonsecaea pedrosoi* var. *phialophorica)*, is an olive-black to brownish-black mold which produces short, velvety aerial mycelium (Fig. 427). On Sabouraud's medium the colony is at first dome-shaped, but becomes flat as the colony slowly increases in size. Sporulation occurs in short, tubular- to flask-shaped cells called "phialides," in the flaring mouths of which colorless thin-walled spores, 1.5 to 2 by 2 to 3 microns in size, are budded off successively. Under certain conditions of culture these conidia increase considerably in size after their abstriction. They remain in a ball-like cluster at the mouth of the phialide if undisturbed. Rarely some strains, otherwise typical of *P. verrucosa*, produce branching conidiophores which bear chains of spores in a manner typical of the following species (Fig. 428).

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[1] Carrión, A. L., and Silva, M.: Chromoblastomycosis and Its Etiologic Fungi, in Biology of Pathogenic Fungi, W. J. Nickerson, ed. Waltham, Mass., 1947, Chronica Botanica Co.

Phialophora pedrosoi (Brumpt) Cif. and Red., 1944 *(Hormodendrum pedrosoi, Fonsecaea pedrosoi)*, produces a colony which is indistinguishable from that of *P. verrucosa,* but the conidiophores bear brown spores laterally or in branching chains (Fig. 429). Most strains also produce a few spores exactly like those of *P. verrucosa* (Fig. 430). *P. compactum* is similar to *P. pedrosoi* except that spore chains are short and less easily dissociated. It has been isolated only a few times.

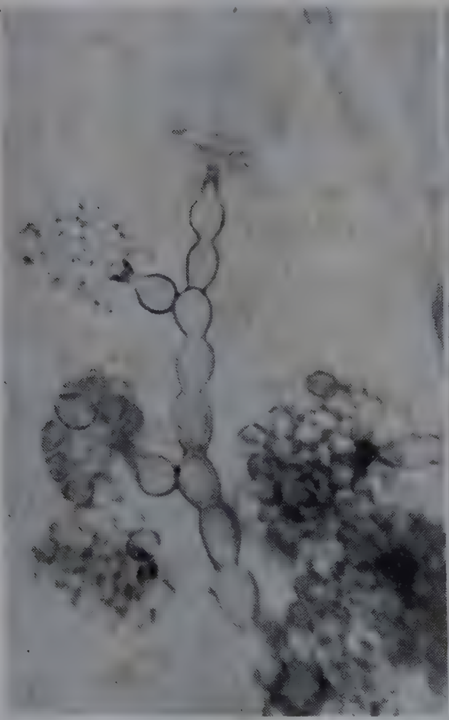

Fig. 428.

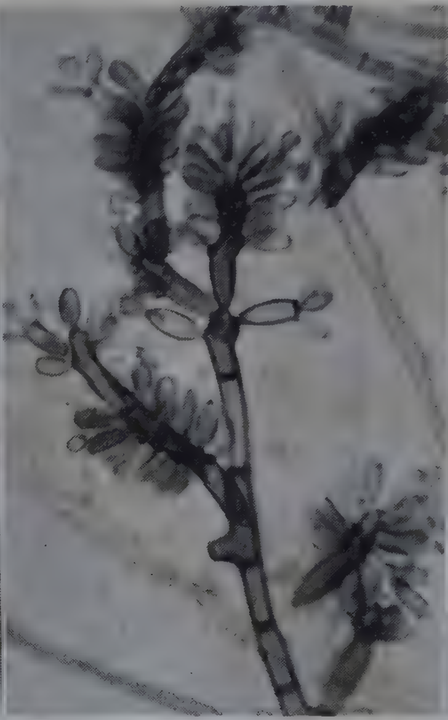

Fig. 429.

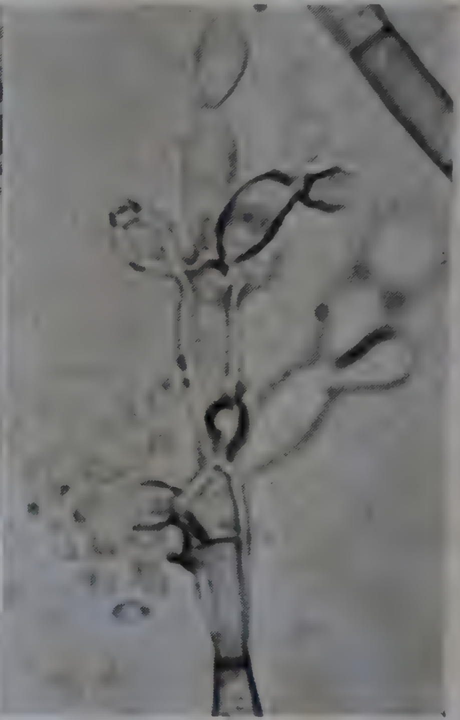

Fig. 430.

Fig. 428.—Phialides (conidiophores of *Phialophora verrucosa.* (×500.) (From Emmons and Carrión: Puerto Rico J. Pub. Health Trop. Med. 11: 703, 1936.)

Fig. 429.—*Phialophora pedrosoi* conidiophores. (×500.)

Fig. 430.—Conidiophore of *Phialophora pedrosoi.* (×500.) (From Emmons and Carrión: Mycologia 29: 237, 1937.)

SPOROTRICHOSIS

The lesion of sporotrichosis develops as a subcutaneous, ulcerating nodule at the site of a thorn puncture. Secondary lesions develop in the lymph nodes draining the area. The disease has been reported frequently in small epidemics in men handling thorny plants. Several thousand cases were observed in laborers in gold mines in South Africa; the etiological agent was easily isolated from the timbers of the mines.[1]

Sporotrichum schenckii (Hektoen and Perkins) Matruchot, 1910, is present in tissues in the form of spherical- to cigar-shaped cells which reproduce by budding (Fig. 431). It is variable with respect to the Gram stain and is so infrequent in human lesions as to be difficult to demonstrate except by a selective stain such as Bauer or Hotchkiss-McManus. In culture, *S. schenckii* produces a flat, sometimes wrinkled or radially folded, usually almost glabrous, brown to black colony. Some strains develop a short aerial mycelium and many strains lose their pigment-producing ability after several subcultures.

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[1] Sporotrichosis Infection on Mines of the Witwatersrand, Proceedings of Transvaal Mine Medical Officers Association, Transvaal Chamber of Mines, Johannesburg, 1947.

Conidiophores are delicate, tapering, lateral branches which bear at the tip a cluster of elliptical colorless conidia 2 to 4 by 2 to 6 microns in size. Sporulation continues toward the base and eventually occurs on undifferentiated hyphae. The conidia, after falling from the conidiophore, are characterized by a tiny apiculus at the point of former attachment to the conidiophore, and careful examination under high magnification will reveal scars on the conidiophore marking points which bore conidia.

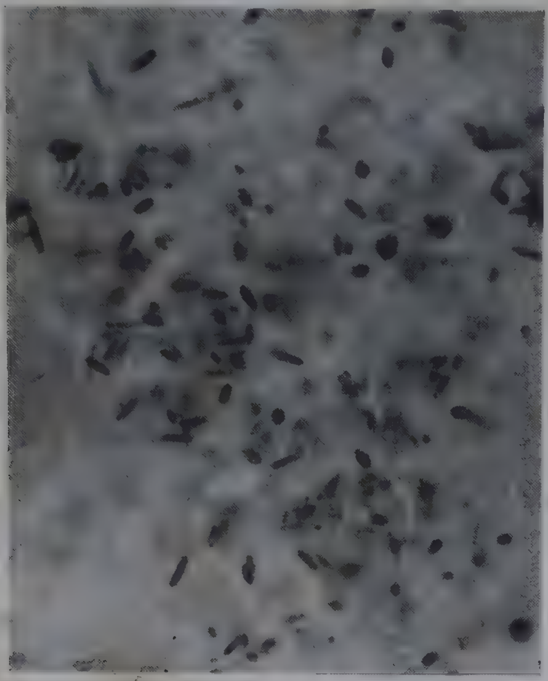

Fig. 431.—*Sporotrichum schenckii* in testis of experimentally infected rat. (×400.)

CRYPTOCOCCOSIS

Cryptococcic meningitis (Torula meningitis) is the most important form of cryptococcosis although the disease may become generalized and involve many tissues. Evidence suggests that the primary lesion is usually a small, inapparent, pulmonary granuloma which, in most cases, heals without sequelae.

Cryptococcus neoformans (Sanfelice) Vuillemin, 1901, *(Torula histolytica)*, can be demonstrated in spinal fluid (where it may be mistaken for a lymphocyte) by mixing the spinal fluid with dilute India ink. This makes the characteristic wide extracellular capsule of the spherical budding cell easily apparent. It can be seen also in the thick myxomatous pus from an abscess where it grows in great numbers, almost as in a culture. Its growth in such lesions and its wide capsule create an appearance in tissue sections which was once erroneously interpreted as lysis of host tissue.

In culture, *C. neoformans* produces a cream- to tan-colored moist colony which may be sufficiently mucoid to flow to the bottom of the slant. Growth occurs at 37° C. but is somewhat more rapid in most strains at 30° C. In culture, as in tissue, the cell is spherical, 4 to 15 microns in diameter, and reproduces by budding. Unlike a typical yeast which is egg-shaped and produces buds near the small end, *C. neoformans* remains spherical even when budding, and when young, produces buds at any point on its surface.

Fungi morphologically indistinguishable from *C. neoformans* are sometimes isolated from sputum. They can be differentiated by lack of virulence for mice, as mentioned previously.

HISTOPLASMOSIS

Histoplasmosis is a disease of the reticuloendothelial system and, unlike most other mycoses, the fungus causing it is predominantly intracellular. The disease is characterized by fever, emaciation, splenomegaly, hepatomegaly, lymphadenopathy, and ulcers of the mucous membranes and sometimes of the skin. Some or all of these signs and symptoms may be present in the generalized form of the disease which has a high mortality rate. It is apparent, from epidemiologic studies employing intradermal tests with histoplasmin and serologic and roentgenologic technics and by the study of small epidemics, that the majority of infections are inapparent or mild and self-limited, involving the lungs or thoracic lymph nodes. In eastern central United States, histoplasmin sensitivity is prevalent, varying according to locality from 30 to 90 per cent in the general population above two years. The curve representing the incidence of skin sensitivity, unlike that for tuberculin sensitivity, rises sharply in preschool and early school years. In the same geographic areas there is a high incidence of nontuberculous pulmonary calcification which may be related to benign histoplasmosis.

The histoplasmin intradermal test has been useful in survey and epidemiologic studies but is of limited diagnostic value in a geographic area where 85 per cent of the population react to the test. The complement fixation test may be of diagnostic value if a rise in titer can be demonstrated. Both the intradermal and serologic tests must be interpreted in light of the fact that there are cross-reactions with blastomycosis and to a lesser degree with other mycoses.

The relationship between geographic areas of prevalent histoplasmin sensitivity and the actual geographic distribution of histoplasmosis is not yet fully elucidated. Cases of histoplasmosis have been observed in many parts of the United States and of the world where the level of histoplasmin sensitivity is not high. This may well be due to a highly localized distribution of the etiological agent, *Histoplasma capsulatum,* which has a saprophytic existence in soil independent of human infection.

The fungus has been isolated many times from soil, first in areas where infected animals had been found and, on several occasions, from soil associated with small epidemics. Many of these epidemics were in men exposed to soil or dust containing excreta of chickens or pigeons, and the fungus has been isolated more frequently from soil collected around old chicken houses than from other sites. However, chickens are not known to be hosts of Histoplasma and are not easily infected experimentally. The relationship appears to be one of a suitable environment for saprophytic growth and not a host-parasite relationship.

Dogs, cats, rats, mice, opossums, fox, raccoons, and skunks have been found with histoplasmosis. Benign histoplasmosis is actually a frequent disease in dogs and cats in some endemic areas. In one such area where an extensive study was conducted, approximately 50 per cent of dogs were proved to have histoplasmosis by isolation of *H. capsulatum*. The hilar lymph nodes were most frequently involved and in some animals the fungus was isolated from no other tissue.

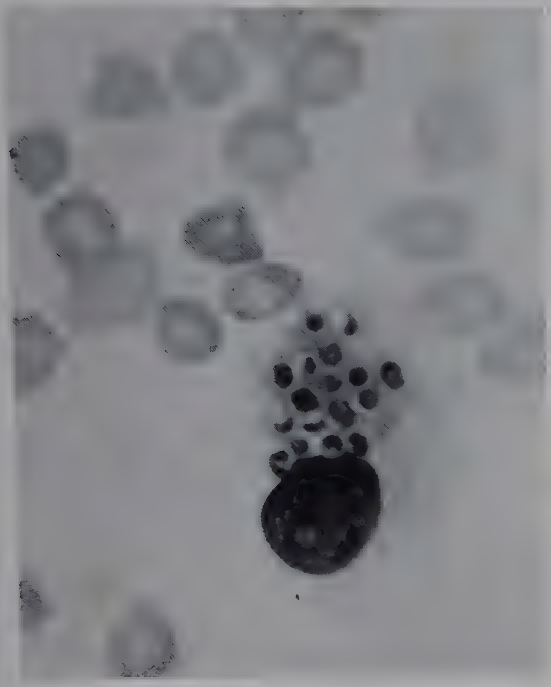

Fig. 432.

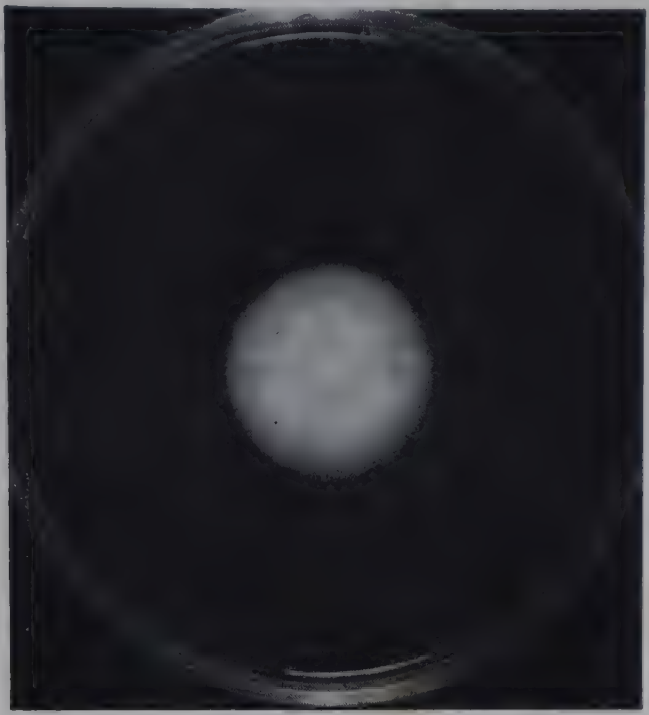

Fig. 433.

Fig. 432.—*Histoplasma capsulatum* in impression smear of spleen of experimentally infected mouse. (×1,000.)

Fig. 433.—*Histoplasma capsulatum* colony incubated 2 weeks at 30°C. on Sabouraud's agar.

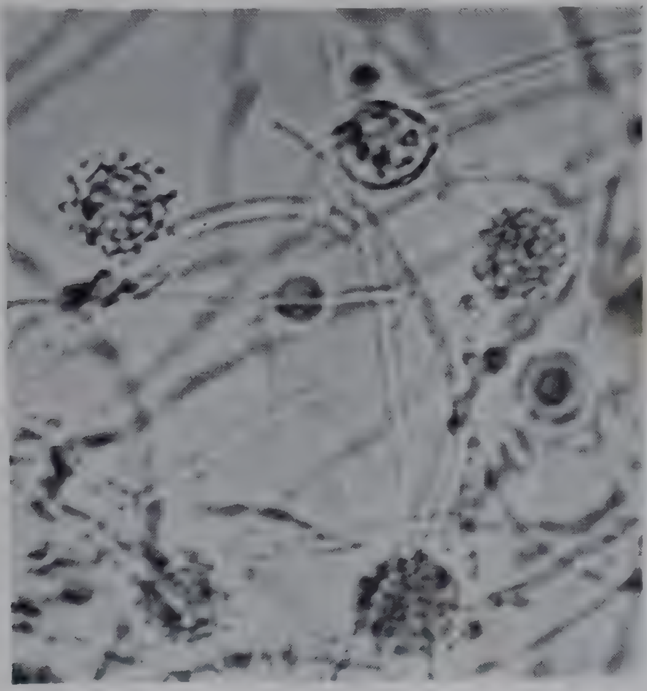

Fig. 434.—*Histoplasma capsulatum* macroconidia. (×600.)

Histoplasma capsulatum Darling, 1906, is a yeastlike fungus 2 to 3 by 3 to 4 microns (occasionally larger) found usually in monocytes (Fig. 432). It can be demonstrated by Giemsa or Schiff staining. Sputum, blood, bone marrow, excised lymph nodes, and scrapings from ulcers are suitable materials for microscopic examination and culture. Attempts to demonstrate and isolate the fungus from any of these sources are usually ineffective in mild cases.

The yeast or parasitic growth phase of *H. capsulatum* can be maintained in vitro by incubation at 37.5° C. on cystine-dextrose-blood agar. When cultures are made on Sabouraud's medium and incubated at 30° C. or room temperature, the fungus grows as a mold and produces a white to brown colony (Fig. 433) which bears two types of spores on short lateral branches. Conidia are spherical, 2.5 to 4 microns in diameter, with a minutely spiny wall. Macroconidia are spherical (rarely clavate), 8 to 12 microns in diameter with conspicuous fingerlike appendages on the surface (Fig. 434). After subculture many strains lose the ability to produce macroconidia. When a suspension of the mold is injected into a mouse or other suitable laboratory animal, the mold reverts at once to the yeastlike phase of growth.

The technic of animal inoculation for primary isolation from man is especially useful in histoplasmosis because of the slow growth of *H. capsulatum,* the suppression of growth by many contaminants, and the susceptibility of mice to a small inoculum. When cultures must be made from sputum, the use of a culture medium containing penicillin and streptomycin will inhibit most bacterial growth but will not inhibit contaminating fungi which often grow rapidly in sputum cultures. Contaminated material of this type can be injected into the peritoneal cavity of the mouse. The mouse should be killed in 3 or 4 weeks and cultures made on Sabouraud's agar from the spleen and liver.

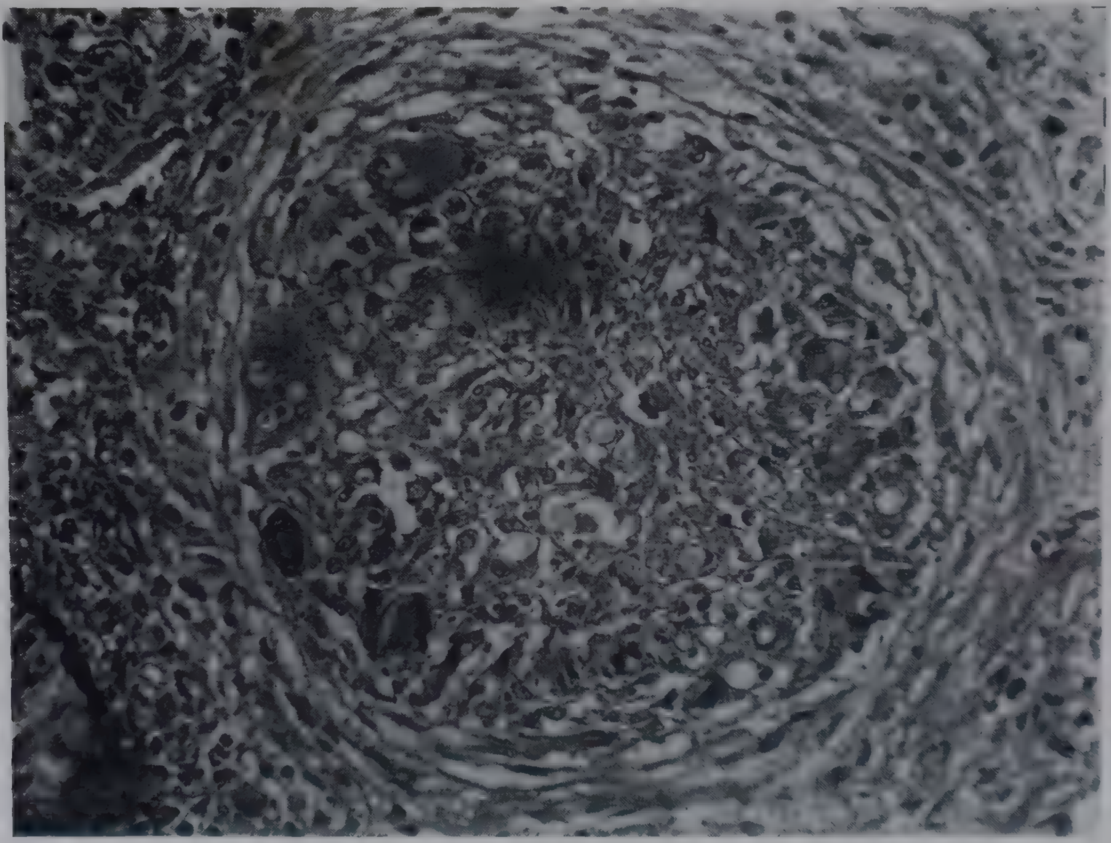

Fig. 435.—Blastomycosis of the lung, showing nodule with giant cells, *Blastomyces dermatitidis.* (Courtesy Dr. Oscar Felsenfeld.)

NORTH AMERICAN BLASTOMYCOSIS

North American blastomycosis is a systemic granulomatous disease in which the primary lesion may be dermal following traumatic implantation of the fungus, but probably is usually pulmonary. Although lesions may occur in many organs and tissues, the lungs are involved in almost all cases of systemic disease. In dermal lesions there is a characteristic raised border in which microabscesses containing the fungus appear (Fig. 435).

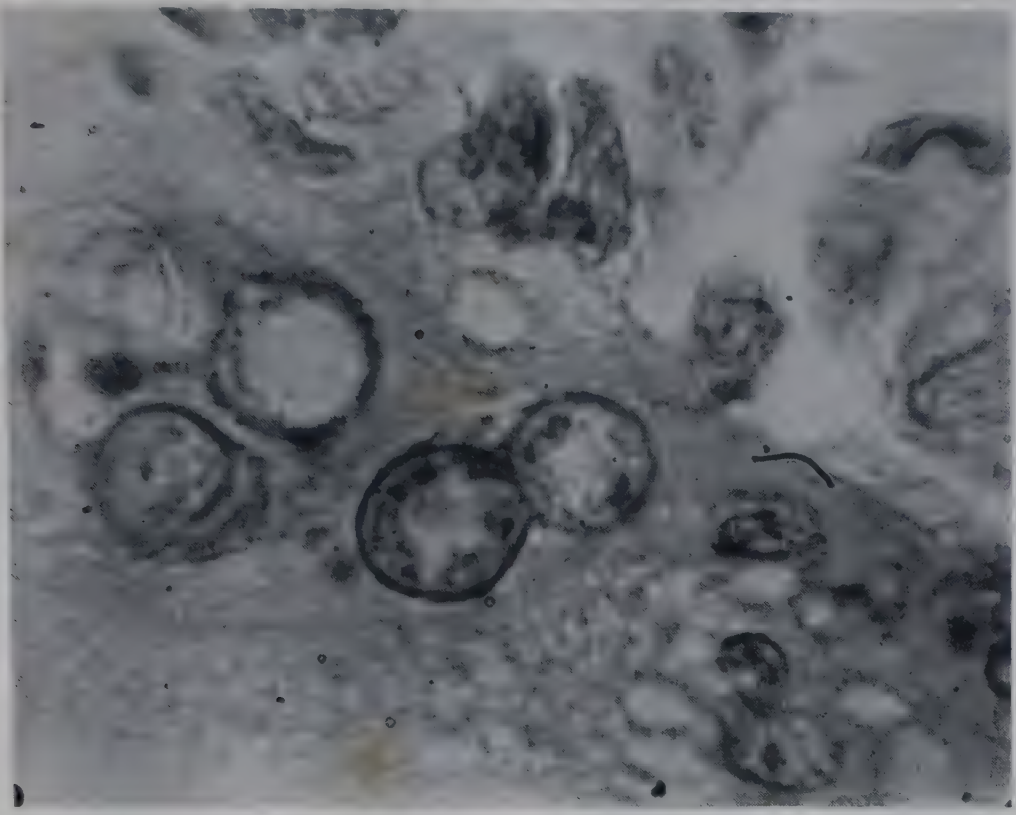

Fig. 436.—*Blastomyces dermatitidis*, budding cell microabscess of the skin. (×900.)

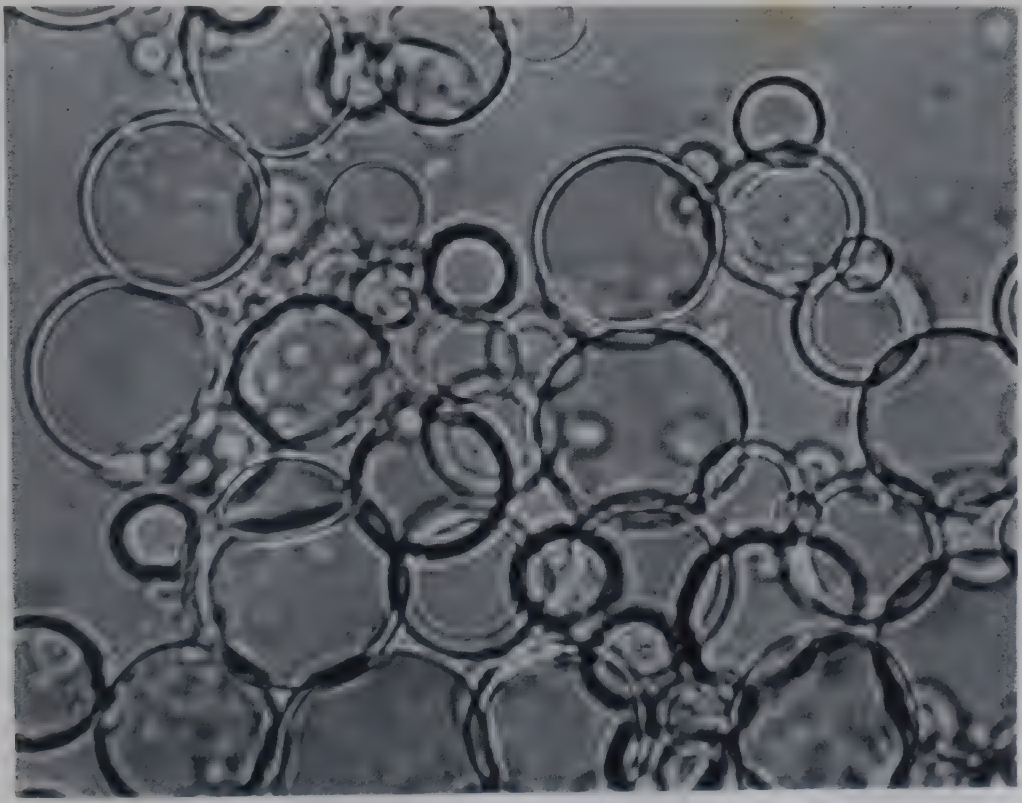

Fig. 437.—Artefact, myelin globules. (×1,000.)

Blastomyces dermatitidis Gilchrist and Stokes, 1898, is a rather thick-walled, yeastlike cell 8 to 12 microns in size. It reproduces by budding, but, unlike Histoplasma, Cryptococcus, and most common yeasts, the daughter bud communicates with the parent cell through a large opening (Fig. 436). Recent reports of small forms of Blastomyces, large forms of Histoplasma, and dual infections by these two fungi emphasize the necessity for a culture before a diagnosis can be made with certainty. Certain artefacts, such as myelin globules which are found in some sputum and tissues, may sometimes be mistaken for yeastlike cells (Fig. 437). Myelin assumes many bizarre budding and tubular forms in wet microscopic preparations. These bodies usually increase in size and number as the preparation stands after treatment with sodium hydroxide.

B. dermatitidis can be maintained in vitro in the yeastlike phase of growth by incubation at 37.5° C. on blood agar, but at 30° C. it is a white to brownish mold. It resembles Histoplasma, but does not produce two distinct types of conidia, and its conidia, which are 2 to 6 microns in diameter, have smooth walls.

SOUTH AMERICAN BLASTOMYCOSIS

South American blastomycosis is a frequent mycosis with a high mortality rate in Brazil and occurs less frequently in other South American countries. Unlike North American blastomycosis, primary lesions are most frequently seen on the oral or nasal mucosa, and involvement of the gastrointestinal tract and lymph nodes is common.

Blastomyces brasiliensis (Splendore) Conant and Howell, 1941, reproduces in the tissues by multiple budding and under certain conditions many small buds are produced simultaneously. As in the case of *B. dermatitidis* the tissue phase can be maintained in vitro by incubation at 37.5° C., but the fungus grows as a mold at lower temperatures. Sporulation is irregular and scanty and is usually lacking in old stock cultures.

COCCIDIOIDOMYCOSIS

Coccidioidomycosis occurs in the arid Southwest of the United States, in northern Mexico, and in other arid regions of Central and South America. Both a benign and a severe systemic form occur, the benign form varying in severity from an inapparent infection to an acute respiratory infection characterized by fever, pleuritic pains, erythema nodosum, and the development of pulmonary cavities. This form is popularly known as San Joaquin Valley fever, desert rheumatism, etc. The severe form is a granulomatous disease of protean manifestations involving almost any organ of the body, and it has a high mortality rate. This form, coccidioidal granuloma, develops more often in Negroes, and other dark-skinned races, than in whites. In general, coccidioidomycosis assumes greatest clinical importance when a susceptible adult population enters an endemic area. In a stable native population it is for the most part an inapparent disease of childhood which appears to immunize against reinfection.[1]

Coccidioidomycosis occurs in dogs, cattle, sheep, and rodents. Apparently these animals as well as man are infected by inhalation of spores of the fungus which have grown saprophytically in the environment. The disease is not contagious. The fungus has been isolated from soil and, like other fungi causing systemic mycoses, is capable of saprophytic growth in soil.

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[1] Smith, C. E.: M. Clin. North America 27: 790, 1943.

Coccidioides immitis Rixford and Gilchrist, 1896, is present in tissue in the form of spherical cells which reproduce by a process called "progressive cleavage" apparently identical to that seen in the sporangium of Phycomycetes. At maturity the protoplasm is divided by cleavage furrows into multinucleate masses which normally are then subdivided by walls into the definitive uninucleate spores, 2 to 3 microns in diameter. The wall of the sporan-

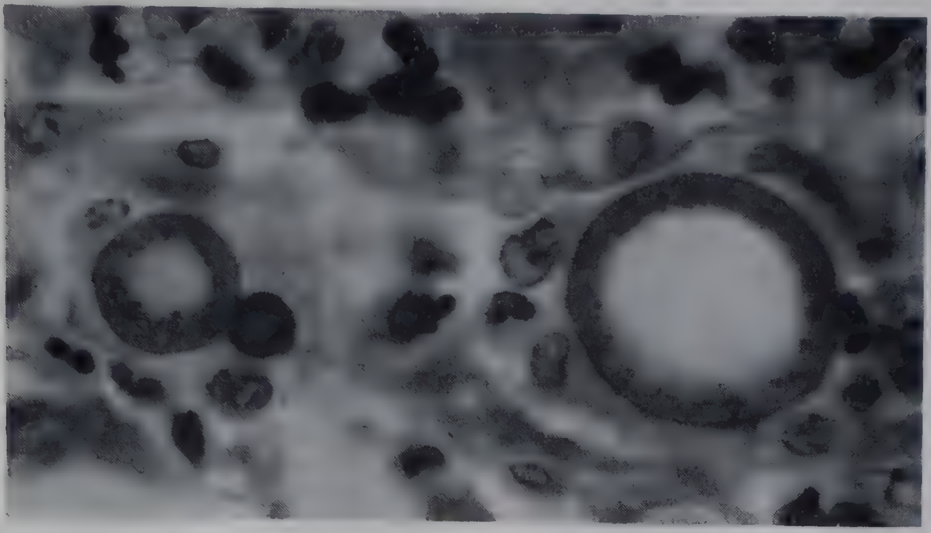

Fig. 438.—*Coccidioides immitis,* three young cells of the fungus in experimentally infected guinea pig. (×1,000; reduced ¼.) (From Emmons: Mycologia 34: 452, 1942.)

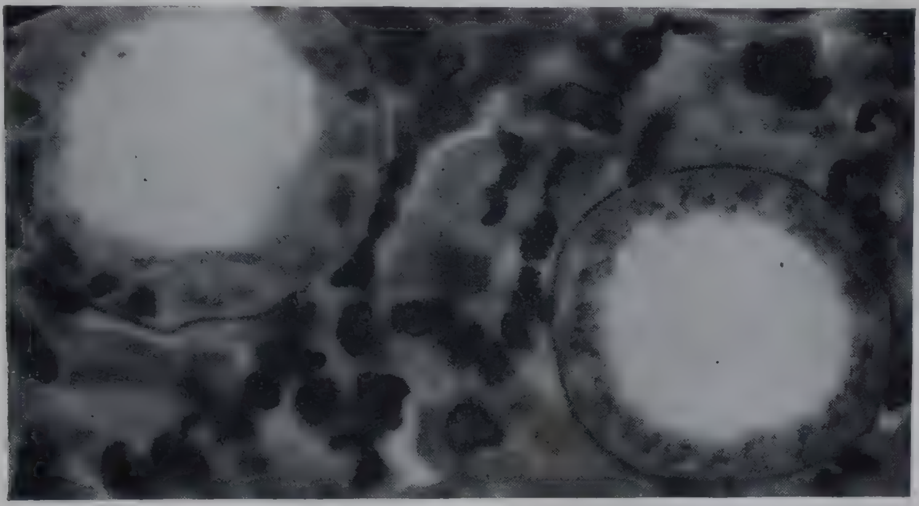

Fig. 439.—*Coccidioides immitis,* large vegetative cell and beginning of sporulation by "cleavage furrows." (From Emmons: Mycologia 34: 452, 1942.) (×1,000; reduced ¼.)

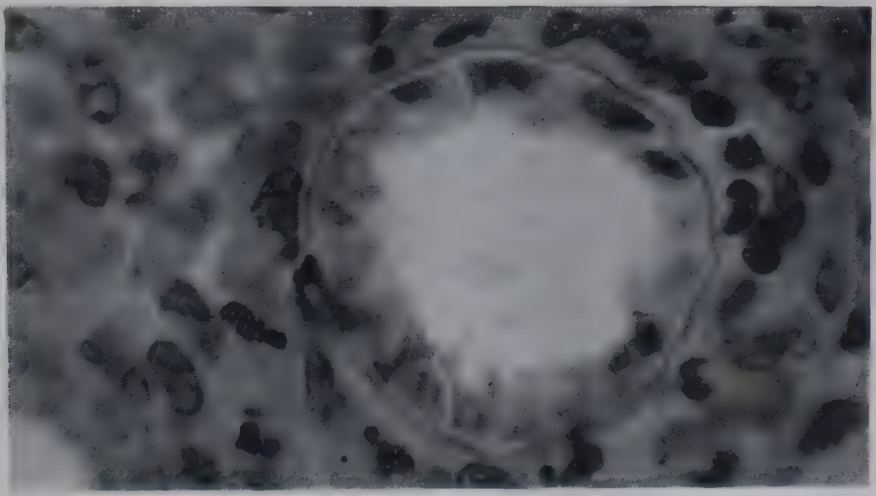

Fig. 440.—*Coccidioides immitis,* early stage in sporulation. (×1,000; reduced ¼.) (From Emmons: Mycologia 34: 452, 1942.)

gium then ruptures and the spores are liberated and grow to reach a mature size of 30 to 200 microns, depending upon the tissue and host species (Figs. 438 to 442). When Coccidioides grows in the wall lining a pulmonary cavity it sometimes produces a mycelium and arthrospores.

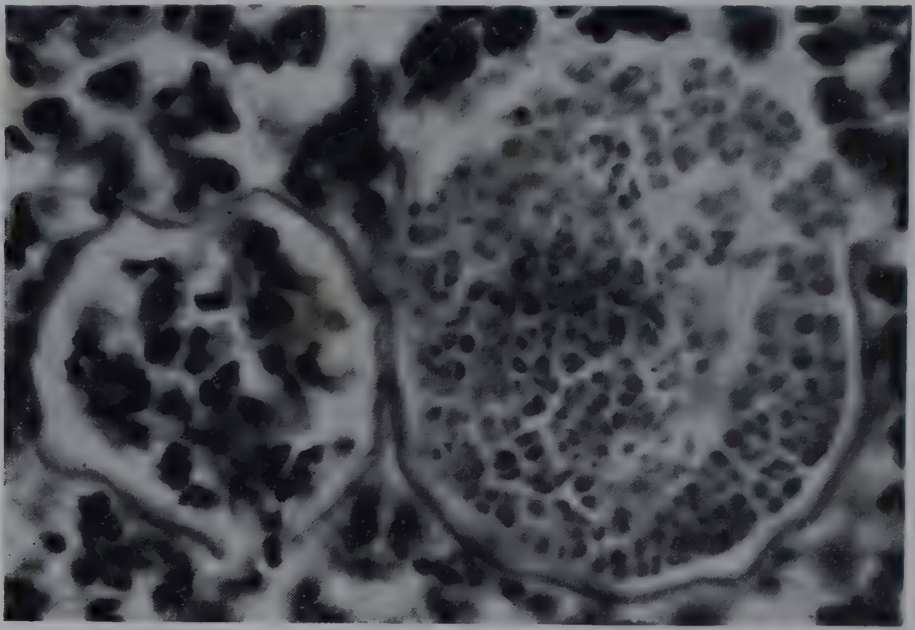

Fig. 441.—*Coccidioides immitis,* mature, ruptured sporangium and old emptied sporangium. (×1,000; reduced ¼.) (From Emmons: Mycología 34: 452, 1942.)

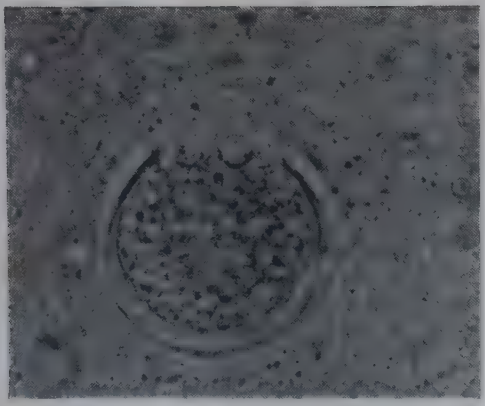

Fig. 442.

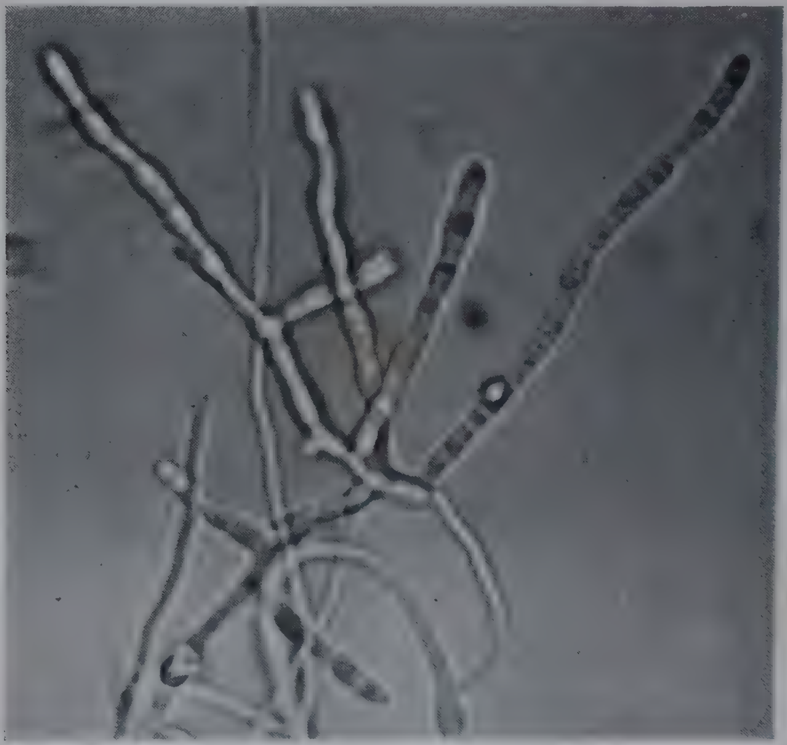

Fig. 443.

Fig. 442.—*Coccidioides immitis,* mature sporangium in pus. (×500.)

Fig. 443.—*Coccidioides immitis,* early sporulation in culture. (×1,000; reduced ¼.) (From Emmons: Mycología 34: 452, 1942.)

C. immitis is easily isolated in culture because of its rapid growth, even in the presence of other molds and bacteria. It grows at 37° C. but optimum temperature is lower and room temperature is adequate. In culture, *C. immitis* is a floccose, gray mold which produces enormous numbers of arthrospores 2.5 to 3 by 3 to 4 microns. The first arthrospores to appear in a culture (at 8 to 10 days) are borne on morphologically differentiated thick lateral branches, but eventually many undifferentiated hyphae sporulate by septation (Fig. 443). There is a characteristic condensation of cytoplasm which results in alternating spores and empty spaces in the hyphae. These arthrospores are easily dissociated and constitute a grave hazard in the laboratory. *C. immitis* should never be grown in plate culture and slant cultures should be manipulated under a hood.

ASPERGILLOSIS

Pulmonary aspergillosis, caused by *Aspergillus fumigatus,* is clinically important although one opinion holds that it is often secondary to another infection or to some anatomic anomaly. The fungus grows in fairly dense colonies in the lung (although it does not form organized granules as in mycetoma), may produce cavities, and characteristic hyphal fragments may be found in great numbers in sputum when one of these lesions communicates with a bronchus (Fig. 444).

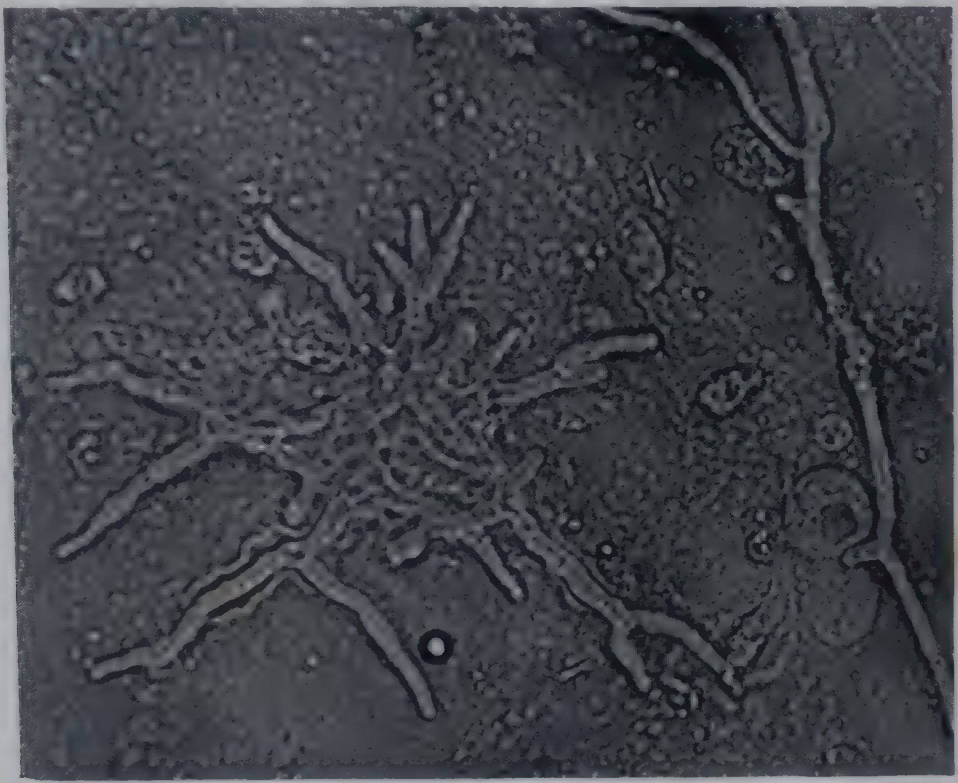

Fig. 444.—*Aspergillus fumigatus,* hyphae in sputum. (×1,000.)

A. fumigatus produces a rapidly spreading gray-green colony. One feature of the conidial heads differentiating them from other species of Aspergillus is the parallel orientation of the sterigmata which results in production of a columnar mass of conidia.

A. niger, the second most important species clinically, is a frequent cause of otomycosis. It is black in culture.

MUCORMYCOSIS

The diagnosis of mucormycosis is frequently made post mortem and is based upon the finding of wide, rarely septate hyphae in the tissues, usually of the central nervous system. In the absence of a culture, a specific diagnosis cannot be made, but the most frequently incriminated species is *Absidia corymbifera (Mucor corymbifer).*

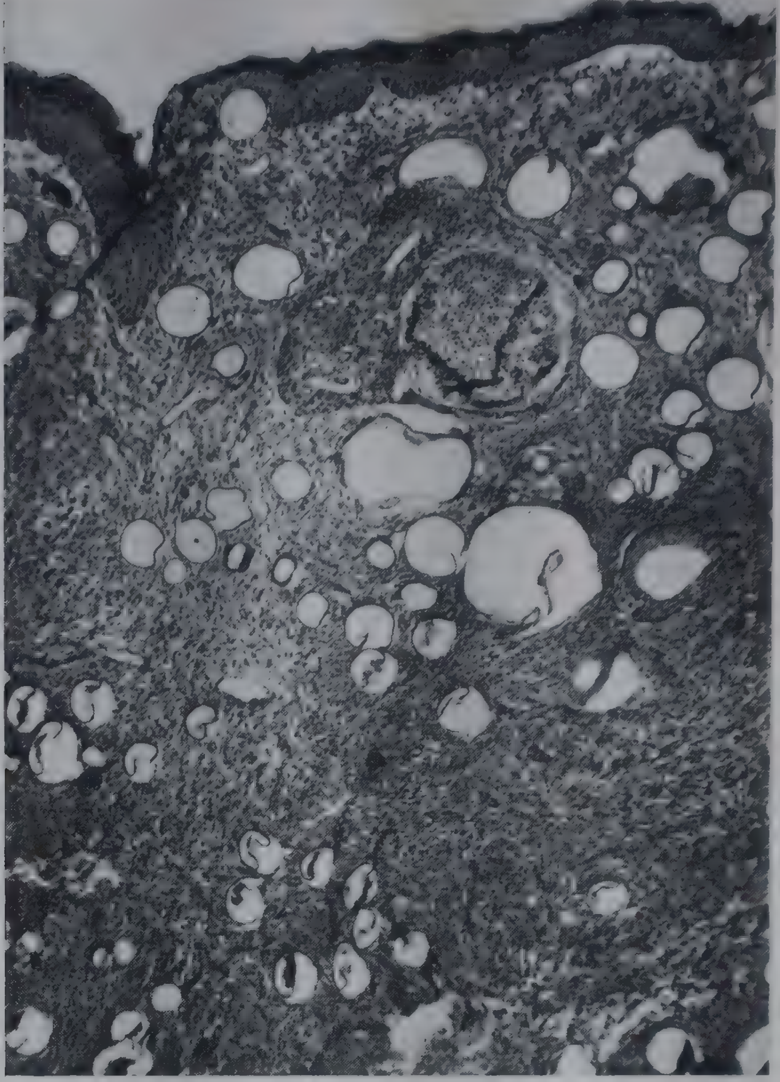

Fig. 445.—*Rhinosporidium seeberi.* View of a nasal polyp, showing one adult parasite and many young stages. The others are deformed, due to action of reagents. (Courtesy Dr. Juan Boggino, Paraguay.)

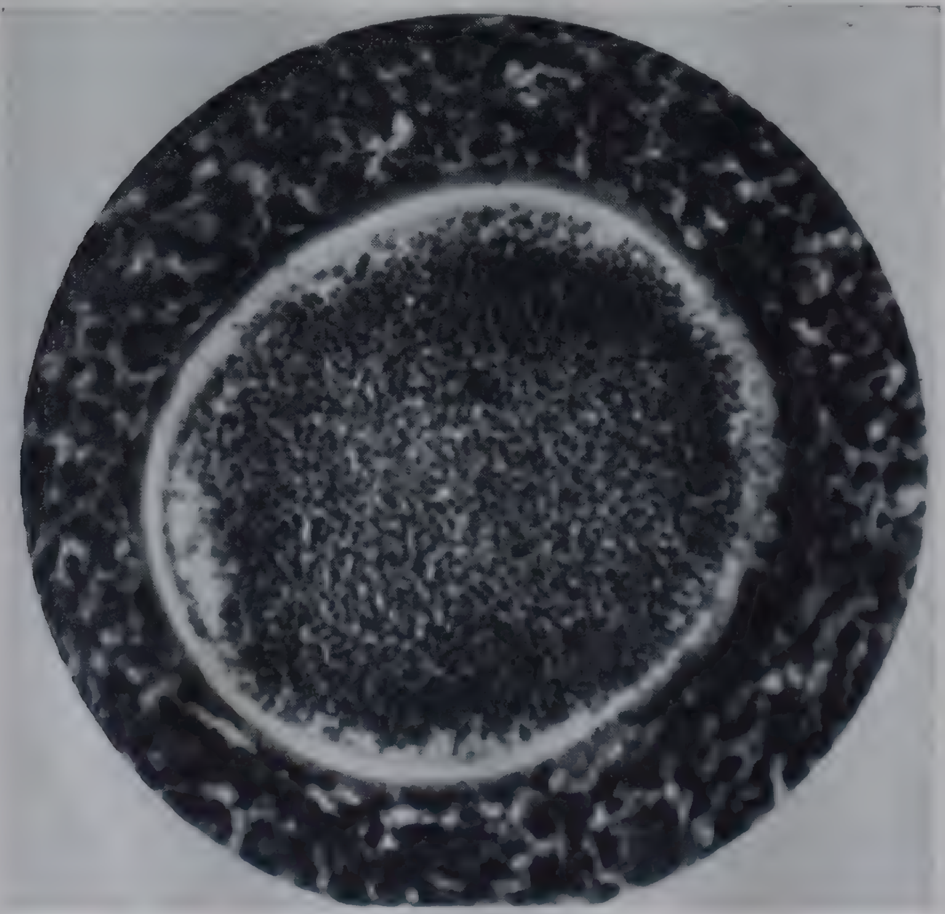

Fig. 446.—*Rhinosporidium seeberi.* A parasite which has almost completed its growth. (Courtesy Dr. Juan Boggino, Paraguay.)

RHINOSPORIDIOSIS

Rhinosporidiosis is a rare, chronic, benign disease of man, cows, and horses. It is manifested as polypoid granulomata of the nasal mucosa and, less frequently, of other mucosal surfaces or of skin.

Rhinosporidium seeberi (Wernicke) Minchin and Fantham, 1905, was thought to be a protozoan until Ashworth advanced the opinion that it is a fungus. It has not been grown in culture and its taxonomic position is still in doubt. In tissue it is a spherical cell reaching a diameter of 300 microns which, at maturity, becomes filled with sporelike bodies (Figs. 445, 446).

CHAPTER XII

PARASITOLOGY AND TROPICAL MEDICINE*

Definitions

Parasitology is the study of parasites. **Parasites** are any living beings, vegetable or animal, which live in part or throughout their entire existence on the surface of or within other living organisms naturally stronger than the parasites. They breed at the expense of the **host,** which is the living organism that harbors the parasites. Hosts may belong to either the animal or vegetable kingdom. The type of host determines the division of parasitology into animal parasitology and vegetable parasitology. Animal parasitology comprises the parasites of animals; vegetable parasitology, parasites of vegetables. This chapter will consider only animal parasitology, including human parasitology.

Parasites may belong to the animal kingdom or to the vegetable kingdom. The first are called **zooparasites,** the latter, **phytoparasites.**

Arthropodology is the study of arthropods. **Arthropods** are characterized by their articulated legs, chitinous exoskeletons, and segmented bodies. Arthropodology may be divided into **acarology,** the study of acarina, and **entomology,** the study of insects.

Grades of Parasitism

There are intermediate phases or grades of parasitism: free, commensal, mutualistic, symbiotic, and parasitism.

Free.—Free-living organisms live on decomposed organic material (**saprophytes**) without establishing any direct relationship to the living body.

Commensal.—Commensal parasitism implies certain advantages of living without causing any damage to the other living animal. This is illustrated by certain small fish which lodge on the outside of certain jelly fish.

Mutualism.—Mutualism means obtaining certain advantages by associating one life with another. An example is the sea crab, which is covered by a sponge. The crab carries the sponge from place to place which helps in clearing the water and in taking on food, and protects it from the voracity of higher animals.

Symbiosis.—Mutualistic animals can live separately, but in symbiosis the two lives are intimately fused and actually constitute a single individual; examples of symbiosis are those lichens formed by the fusion of an alga and a fungus.

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*This chapter has been written with the assistance of Dr. Pedro Kourí, Director. Institute of Tropical Medicine; Professor, Parasitology and Tropical Medicine; Dean, Faculty of Medicine, Havana University, Havana, Cuba; Director, Laboratorios Kuba, Havana, Cuba.

Parasitism.—In parasitism, the parasite derives advantages by living on another entity, the **host,** with which it is associated.

Pseudoparasites

Pseudoparasites or artefacts are macroscopic nonparasitic objects erroneously diagnosed as parasites, or microscopic matter simply in transit through the digestive tract.

Life Cycle of Parasites

Parasites with direct life cycles (one host) are *monoxenous;* those with indirect cycles (more than one host) are called *heteroxenous.* Monoxenous parasites need no intermediate host in their development. Heteroxenous parasites are *diheteroxenous* when requiring only one intermediate host and *polyheteroxenous* when having more than one intermediate host; for example, *Ascaris lumbricoides* is a monoxenous parasite, *Taenia saginata* a diheteroxenous parasite, and *Diphyllobothrium latum* a polyheteroxenous parasite.

Reproduction of Parasites

Reproduction of Protozoa.—Some protozoa reproduce by bipartition and others by gemmation; some by schizogony and others by sporogony; in others, the two processes are alternate, first schizogony and then sporogony: **heterogony**.

In **bipartition,** the protozoa divide into two equal series. This is observed in the amebae, flagellates, and other protozoa. In **gemmation,** common to the lower vegetable forms, such as fungi and some bacteria, the mother cell projects a branch which gives rise to a new element similar to the mother cell.

In **schizogony,** the protozoan divides into various parts. This, like bipartition, is an asexual type of reproduction.

Sporogony is a form of sexual reproduction of the protozoa in which male elements with spermatozoid function are present, as are female elements which are fertilized. The parasites of malaria present both types of reproduction, schizogony and sporogony. Schizogony takes place in the human organism and sporogony in the mosquito. (See pages 1889 ff.)

Reproduction of the Trematodes.—Most of the trematodes are hermaphroditic. Certain types, such as the schistosomas, have separate sexes. In addition to the sexual type of reproduction, the trematodes also have a special type of asexual reproduction, gemmation, which is observed in the larval form, but not in the adult.

Reproduction of the Cestodes.—Cestodes are successive hermaphroditic. Each flatworm shows reproduction by gemmation in the adult form, and partly also in the larval stage.

Reproduction of the Nematodes.—Most of the nematodes are of different sexes. Some, no doubt, like *Strongyloides stercoralis,* have parthenogenetic reproduction, although this has been denied by certain authors.

Reproduction of the Arthropods.—Generally, the arthropods contain both males and females, but some species present normal parthenogenesis and others accidental parthenogenesis with respect to the male.

Dissemination of Parasites

Parasites are widespread in nature. Eggs and larvae of helminths and cysts of protozoa leave their hosts in the feces, sputum, urine, etc., and abound in nature. They contaminate water and vegetation. Man acquires these parasites passively at times by ingesting water, vegetables, and other food contaminated with infestive forms, such as embryonated eggs of nematodes, cysts of protozoa, or through ingestion of intermediate hosts such as the meat of hogs, fish, crabs, etc., which are parasitized by the infective larval forms.

Certain organisms, like the *Wuchereria bancrofti,* emerge actively from their chitinous coverings and bore through the skin. Others are injected into the host by the bite of an insect, such as Plasmodium, Trypanosoma, etc. Some are left upon the skin by hematophagous arthropods at the moment of biting, such as Spirochaetae, Rickettsiae, etc. Other parasites are transmitted by direct contact, such as the treponemata of syphilis and yaws, Sarcoptes, etc.

Eggs and cysts leave the body with the stools, sputum, and urine, to continue their evolution. Certain parasites, however, such as Plasmodium, Trypanosoma, Filaria, cannot continue their life cycles except through the intermediation of hematophagous ectoparasites (mosquitoes, flies, Hemiptera), which extract them from the blood.

There are, in nature, healthy or convalescent ''carriers,'' which are the reservoirs of parasites, and these reservoirs may disseminate and maintain the lives of such parasites.

LABORATORY METHODS FOR THE DIAGNOSIS OF PARASITISM

Laboratory methods for the diagnosis of parasitism can be divided into direct and indirect. Direct methods permit one to see the parasite or the elements from which they are derived; indirect methods include investigating cytologic and immunologic reactions of the invaded organism, such as eosinophilia, antibody formation, etc.

(1) Direct Methods: Absolute Diagnosis

To make an absolute diagnosis of parasitism, it is necessary to see the parasite in any of the stages of its evolution, the eggs, larvae, adults, trophozoites, cysts, or the elements derived from them, liquids, membranes, hooklets, pigments. A simple macroscopic examination is often sufficient in many cases to make an absolute diagnosis. At times it is necessary to magnify the objects found. For this purpose, simple lenses or binocular stereoscopic microscopes or compound microscopes with high magnification are used.

Material for examination is quite variable, and it is necessary to carry out various procedures: examination of the sputum, nasal secretions, gastric juice, duodenal fluid, feces, rectal secretion, urine; material obtained by biopsy, at autopsy, in the operating room, or by puncture; spreads made from the spleen, sternum, cystic liquids, abscess pus, exudates from ulcers, etc. Examination of bile obtained by duodenal intubation is very important in the absolute diagnosis of certain parasitoses of the biliary or intestinal channels.

An outline of direct methods is given in Table 172.

TABLE 172

- Direct Method Absolute Diagnosis
  - Macroscopic examination or examination with slight magnification
    - by simply looking at it
    - with a hand lens
    - with binocular stereoscopic microscope
    - screening of the feces
  - Microscopic examinations
    - direct preparations
      - dark-field condenser (ultramicroscopic)
      - bright-field condenser
        - examination of fresh specimen or by stain of moist specimen
        - examination by staining fixed smears
    - concentration methods
      - mechanical
        - blood
          - thick drop
          - centrifugation
        - stools
          - solutions of high density
          - solutions of low density
      - biological
        - coprocultures: hydrotropism, phototropism
        - xenocultures: by reproduction, by concentration
        - other cultures: blood, excreta, exudates, etc.
        - experimental inoculation

Macroscopic Examination.—Certain parasites can be seen macroscopically. Parasites of *Taenia saginata,* the most frequent parasite of man, can be identified in most cases by finding proglottids, which emerge actively from the anus. These proglottids may emerge alone or admixed with fecal material. *Enterobius vermicularis* can be seen frequently with the naked eye in the stools of parasitized individuals. Sometimes the patients bring for identification parasites which they have seen in their stools, on their bodies, or on their underclothing.

In macroscopic examination of pus from cutaneous fistulas there may be seen characteristic yellow granules of actinomycosis which can be confirmed by direct microscopic examination of the fresh specimen between cover glass and slide.

Most of the larger ectoparasites are recognized by simple macroscopic examination or by the use of a hand lens or binocular stereoscopic microscope. These are such organisms as larvae of myiasis, pediculi (*capitis, vestimenti,* and *pubis*), anopheles, reduvidae, ixodidae, etc.

Ascaris lumbricoides may be expelled by mouth or at the anus and can be recognized at a glance.

Screening.—At times the segments of Taenia or the oviferous female of Enterobius are found mixed in stools and in order to see them it may be necessary to dilute and screen the fecal material.

If the stools are not liquid, dilute with water in the same container.

Agitate vigorously and pass them through a collander with 1 mm. apertures.

Wash them freely in running water and then place the collander over a bucket with a flat bottom, white or black, and let the water fall over the outer convex surface of the collander so as to catch all the small particles and parasites retained by it.

When examining the contents of the bucket the parasites can be found if present in the stools. Mucous membranes can also be found, as can vegetable remnants, confused by beginners with true parasites (pseudoparasites).

For the identification of these elements, lenses or a binocular stereoscope microscope can be used when a simple macroscopic examination is insufficient to identify them.

Microscopic Examination.—Examine a drop of the fresh material, stools, blood, pus, etc., between cover glass and slide, for microscopic examination for eggs and larvae of intestinal parasites, embryos of filaria, trypanosomes and plasmodia in blood, protozoa and their cysts, fungi, etc. At times it is necessary to add to a drop of the fresh material a drop of strong Lugol solution, as in the study of intestinal protozoa and their cysts, or a Lugol-eosin mixture, or weak solutions of supravital stains like neutral red, methylene blue, brilliant cresyl blue, etc.

In affections of the skin and its appendages the examination is made of scales, nails, hair, etc., mixed with one or more drops of 40 per cent potassium or sodium hydroxide, using the high-power magnification of the microscope. Mycelial filaments, spores, etc., can be seen.

Examination of Stained Spreads.—Giemsa stain diluted in the proportion of 1 or 2 drops of the stain to each c.c. of neutral or slightly alkaline distilled water can be used to stain and demonstrate the finer elements of blood or visceral spreads, after methyl alcohol fixation, or for the staining of the thick drop of blood which has been previously hemolyzed. The thick drop is used for examination for parasites of malaria, trypanosoma, leishmania, filaria, etc.

Staining by Gerlach or van Gieson methods of spreads of Ammon's horn as well as silver impregnations of frozen sections of these nerve structures and staining by Ziehl-Neelsen method of paraffin sections of Ammon's horn will demonstrate Negri bodies in rabies. Such preparations are examined with the oil immersion system.

Heidenhain's iron-hematoxylin and its modifications as well as the Mann method for staining spreads of stools or of muco-pus of moist specimens fixed with mercurial fixatives are used in the study of the finer structures of intestinal protozoa and their cysts. This method is at times indispensable in making a differential diagnosis of the various species. Gram's method should be used systematically in the investigation of intestinal protozoa. This is an excellent method for diagnosis as noted in the publications of Kourí and Basnuevo. Silver impregnation is used* for the study of spirochetes in general, especially the *Treponema pallidum* and *T. pertenue*. A dark-field examination of the sera from such lesions should also be made.

Concentration Methods

When the parasites and elements derived from them are present in fluids such as blood, urine, bile, or in excretions, it is necessary to use concentration methods to demonstrate them. There are mechanical and biological concentration methods. Mechanical methods are applicable to blood parasites and biological methods to intestinal parasites.

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*Krajian method is recommended.

Mechanical Methods.—Blood.—The thick drop of blood hemolyzed and stained with Giemsa stain can be considered a concentration method for the investigation of malaria plasmodia, trypanosomata, leishmanias, spirochetes of relapsing fever, microfilariae, etc. Other methods of concentration of parasites in blood are based upon centrifugation of large quantities of blood either hemolyzed or not hemolyzed, for the investigation of microfilariae and trypanosomes.

Stools.—There are many concentration methods for eggs, larvae, and cysts. All are based on the use of a large amount of fecal material which is rendered homogeneous with water or saline solutions and screened. This separates the large quantity of thick solid material which is retained upon the screen. Cysts, eggs, and fine forms pass through the screen and are collected into a conical tube and centrifuged. They then can be found in the sediment. Some authors—Usami and Kamada—have demonstrated that the screen may hold back a large number of eggs and cysts and that it is necessary to examine the thick particles which fall to the bottom, decanting the supernatant fluid. This should be repeated several times.

Many concentration methods are carried out with solutions of a lower density than that of eggs and cysts (see pages 1811 and 1812), concentrating the eggs and cysts at the bottom of the tube; others use solutions of a higher density than that of the eggs, the upper portion of the fluid then being examined.

Biological Methods.—Coproculture.—Coproculture is carried out for the diagnosis of parasitism of *Strongyloides stercoralis, Necator americanus, Ancylostoma duodenale,* and the schistosomas, or bilharzias, when the larvae and the eggs are held within the stools. Generally for coproculture the stools must be mixed with animal charcoal or with pulverized and sterilized dirt and water, although Kourí's experiences have shown that good results may be obtained without using any of these substances. In coproculture, advantage is taken of the property shown by certain parasites to be attracted by water, positive hydrotropism, or by light, positive phototropism. When a little water at 28° to 32° C. (82° to 90° F.) is directed to a mass of pasty stool, after a certain time if the stools contain larvae of Ancylostoma and Strongyloides these larvae leave the stool and move toward the water. Here they concentrate. Examination of a drop of this fluid will reveal them, whereas direct examination of the stools would not have. Coproculture is simply a method of biological enrichment, based upon the tropism of larvae.

In the case of schistosomiasis with only a few eggs in the stool, if the feces are diluted with water the larvae are set free after a certain time, and accumulate on the surface of the liquid, being attracted toward the more illuminated portion by its phototropism. At times coproculture serves as an enrichment not only for concentration of larval forms by tropism, but also for actual multiplication of the parasites. This is true of the *Strongyloides stercoralis* which completes its sexual cycle in the coprocultures.

Xenoculture (Xenodiagnosis of Brumpt).—Brumpt gave the name of xenodiagnosis to a procedure worked out by him in 1905 for diagnosis of trypanosomiasis of certain vertebrates where the parasites are scarce in the blood. If intermediate habitual or vicarious hosts suck the blood of certain vertebrates, a diagnosis of trypanosomiasis can be made in the vertebrates by finding the evolutionary form of the trypanosomes in the intestines of these intermediate hosts. This procedure is in reality a hemoculture in the digestive tract of the intermediate hosts where these flagellates multiply actively. This is called *xenoculture.* At times multiplication of parasites does not occur in the digestive tract of the host, but instead there is concentration in the extracted or sucked-out blood. This occurs, for example, when the microfilariae which may be found in large numbers in the blood are extracted by the Culex mosquito from the patient bitten by the mosquito. This attraction of embryos of filaria by its intermediate hosts is a means of diagnosis in cases in which the embryos are scarce in blood.

Xenocultures can also be used for differentiation of various spirochetes which are morphologically identical. For example, cosmopolitan relapsing fever is due to the *Borrelia recurrentis* and transmitted by lice; relapsing fever carried by the sheep tick is transmitted by various species of the genus Ornithodorus; African relapsing fever is due to *Bor. duttonii,* and is transmitted by the *Ornithodorus moubata* and by the *O. savigny;* American relapsing fever due to the *Bor. venezuelensis* is propagated by the *O. venezuelensis* (*O. rudis*) and by the *O. talaje;* Spanish relapsing fever due to the *Sp. hispanicum* has for its vector host the *O. marocanus.* All these spirochetes are morphologically identical, but have different vector hosts; they are therefore all biologically different spirochetes.

Other Culture Methods.—Inoculation of blood, stools, and exudates in special culture media is used as a diagnostic method in certain parasitoses.

Blood Culture.—Blood culture is a method of inoculating blood on artificial media and is used as a diagnostic measure in certain protozoal diseases, such as leishmaniasis, sodoku, when these parasites are scarce in the blood.

Culture of Stools.—When intestinal amebiasis is suspected and the direct coprologic examination even with concentration has failed to reveal them, cultures of the stools must be made, using a small portion in special media. As has been pointed out, culture or inoculation of stools is a different mechanism from coproculture because in cultures a small portion of the stool is inoculated to allow the parasites to reproduce, and in coproculture multiplication of parasites does not occur, but a concentration due to tropism. In reality the word coproculture signifies culture of the stools and should be applied to the actual culture. Some other name should be used in place of coproculture, for example, *tropodiagnosis,* because this method is based upon the tropism of the larvae. The stools can be left on the laboratory table at room temperature in the fresh state with the object of studying the development of the eggs of the nematodes, the formation of the embryo, etc.

Exudates.—In suspected lesions of mycosis, leishmaniasis, sodoku, etc., in which direct examination and stained specimens have proved negative, inoculation on special media may successfully make the diagnosis.

Experimental Inoculation.—Another means of diagnosis of parasitic diseases is experimental injection of the suspected material into laboratory animals; for example, kittens and puppies for amebiasis, chicks for intestinal protozoa, rats for sodoku and trypanosomiasis, guinea pigs for icterohaemorrhagic spirochetosis, etc.

Duodenal Intubation.—Macroscopic and microscopic examinations of fluids obtained by duodenal intubation permit the diagnosis of certain parasitoses of the biliary tract and the duodenum with much greater rapidity and security than examination of the fecal material. This has been demonstrated by Bacigalupo, Toullec, and Riou, and Kourí and associates. Distomiasis hepatica by *Fasciola hepatica* and *Clonorchis sinensis,* the lambliases, strongyloidiasis, necatoriasis, etc., are parasitoses in which duodenal intubation renders a great service from the diagnostic point of view. Pseudoparasitism by ingestion of eggs of *F. hepatica* is distinguished from true parasitism by examination of the bile obtained by duodenal intubation. In erratic parasitism, gastric, duodenal, or biliary, with *Ascaris lumbricoides,* microscopic examination of the bile and its flocculent contents can reveal an abnormal localization of this parasite.

Differential diagnosis between *Necator americanus* and *Ancylostoma duodenale* is difficult to prove from the eggs found in the stools, but it is possible if adult forms of the parasite are found and this finding is combined with the examination of bile obtained by duodenal intubation. Occasionally larvae of Strongyloides and the vegetative forms of lamblias are found in the bile and duodenal liquid of individuals whose stools have been negative for these parasites.

(2) Indirect Methods

Indirect methods for the diagnosis of parasitism differ from direct methods in that they do not permit a view of the parasites, but rather an investigation of their effects on the cellular and immunologic reactions of the host in the presence of active and aggressive parasitoses. A definitive diagnosis of the parasitism is not always possible by these methods.

Cellular reactions comprise blood eosinophilia; local eosinophilia; leukopenia with monocytosis; anemia; local inflammatory reactions, acute, subacute, and chronic; remote reactions such as lymphatic gland enlargements, hepatomegaly, changes in the bone marrow; hyperplasia, metaplasia, and neoplasia. Blood eosinophilia in a tropical country makes one think of parasitism, and an investigation for parasites should then be made by direct methods. Anemia might be due to many causes, among them the anemias due to Necator and Ancylostoma. Monocytosis is frequently seen in malaria.

Flocculation Reactions

Various flocculation tests are in use, principally the Kahn, VDRL, Meinecke, Sellek-Frade, Basnuevo, etc.

The Henry-Melano Flocculation Test.—In 1927, Henry[1] presented his melano- and ferro-flocculation tests. He advanced the theory that the

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[1] Gaz. hevd. Sc. méd., Bordeau 48: 311, 1927.

''melanotic'' and ''yellow iron pigments'' formed in the organs of malarial patients were active substances rather than inert deposits; that they were malarial antigens or endoantigens and either gave rise to the formation of endoantibodies or disturbed the colloidal state of the serum in such manner as to impart to it certain flocculating peculiarities. Because true malarial pigment was difficult to obtain, he chose choroidal melanin and certain organic iron compounds as antigens for his test. He regarded the attenuation or entire disappearance of the reaction in sera which were obtained during a paroxysm as being due to the fixation of the endoantibodies, already present, by an overwhelming amount of new endogens produced from the massive destruction of erythrocytes during the attack.

Technic.—

Mix 1 part of serum with 4 parts of a suspension of melanin, formolized at 1:2000, either in distilled water or in 0.3 per cent saline, for sera which flocculate spontaneously in a 1:5 dilution of distilled water.

At the same time prepare serum controls and antigen controls in the usual manner. Stopper the various mixtures and incubate for 3 hours at 37° C.

Allow to stand for 30 minutes at room temperature and read the reaction macroscopically.

Flocculation of melanin indicates a positive reaction. Flocculation in the serum control tube is called by the author ''surflocculance'' and supposedly occurs in some normal sera and nonmalarial sera.

Since the interpretation of the reaction is difficult macroscopically, Henry later advocated the use of the photometer of Vernes, Bricq, and Yvonne, for the quantitative equivalence of the optical density, developed in the tubes during incubation.

The fero-flocculation test, which at first was run concurrently with the melanin reaction, was abandoned by its authors because of its lesser sensitivity and the difficulty of procuring uniform iron preparations.

Proske and Watson Protein Tyrosin Reaction.—Proske and Watson[2] devised a simple accurate colorimetric test obviating the necessity for a photometer. The procedure is based on the fact that proteins possess a chromogenic property which can be measured quantitatively against the color produced by pure tyrosin in the presence of a phenol reagent. This chromogenic value is constant for a given protein, and the intensity of color produced can be used as a measure of the amount of the protein examined.

Serum euglobulin is precipitated from the serum to be examined by adding 13.5 per cent sodium sulphate solution according to the method of Howe. The tyrosin-chromogen index (TI) is determined by comparison with standards prepared from pure tyrosin (Pfanstiehl).

Proske and Watson found in 2,500 normal blood sera that the tyrosin index or euglobulin fluctuated between 50 and 80 while that for sera from malarial patients ranged from 80 to 280 or higher. The test was found to be indicative of the presence of malaria in 97.4 per cent of known malarial cases examined, as compared with 81.9 per cent positive thick blood films examined at the same time.

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[2] Pub. Health Rep. 54: 158, 1939.

Like the Henry test and its modifications, this test is nonspecific, but its high sensitivity in malaria may make it a useful adjunct to the laboratory diagnosis of this disease. It may also be helpful in the differential diagnosis of other pathologic conditions characterized by increase in serum globulin.

Reagents.—

14 Per Cent Sodium Sulphate Solution.—

Dissolve 70 gm. of sodium sulphate, c.p., anhydrous
in 300 c.c. of freshly prepared distilled water.

Dilute to 500 c.c. with distilled water, at a temperature of 37° C. Store in an incubator at 37° C. Solution is stable indefinitely.

5 Normal Sodium Hydroxide.—

Dilute saturated, carbonate-free sodium hydroxide solution to 20 per cent (20 parts per 100).

Tyrosin Standard Solution.—

5 c.c. contain 1 mg. tyrosin. See page 305.

Phenol Reagent of Folin and Ciocalteu.—See page 305.

Technic.—

Unknown.—

Place 3 c.c. of 14% sodium sulphate solution in a small test tube (75 by 100 mm.).

Using an accurately calibrated pipette, add 0.1 c.c. of unheated, clear, nonhemolyzed, nonchylous serum.

Mix by inverting a dozen times, avoiding air bubbles.

Stopper the tube and place in an incubator at 37° C. for 3 hours.

Centrifuge at 1500 r.p.m. for 10 minutes.

Completely pipette off the supernatant fluid.

Wash the precipitate twice with fresh sodium sulphate solution by centrifugation.

This is necessary in order to remove traces of albumin and pseudoglobulin that may have been caught in the precipitate or may adhere to the walls of the tube, and which will also react with the phenol reagent, giving too high readings.

Dissolve the washed precipitate in 1.75 c.c. of distilled water.

Add 0.1 c.c. of 5 normal sodium hydroxide.

Stock Standard.—Use a test tube graduated at 20 c.c.

Place 2 c.c. of the tyrosin solution in the tube.

Add 5 c.c. of distilled water
and 1.0 c.c. of 5 normal sodium hydroxide.

Heat both the unknown and the standard in boiling water for 10 minutes and allow to cool.

Add to the unknown 0.15 c.c. and to the stock standard 1.5 c.c. of the phenol reagent, and dilute the standard to the 20 c.c. mark with distilled water.

While the color is developing, set up a series of small tubes, 75 by 10 mm., and mark the tubes with a wax pencil 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10. Prepare *substandards* in these tubes according to the following schema:

Substandards—per cent	100	90	80	70	60	50	40	30	20	10
Stock standards—c.c.	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2
Distilled water—c.c.	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8

Compare the color intensity of the unknown with these standards. If the color of the unknown falls between two whole gradations, the color value is intermediate between the two. For example, if the unknown falls between 60 and 50, then the reading is 55.

Observe the following precautions: All glassware must be chemically clean, but sterility is unnecessary. The serum pipettes should have fine tips, because small droplets of serum adhering to blunt tips may cause considerable error. Sera should be clear. Hemolyzed sera give too high tyrosin values owing to their globin content; chylous sera give too low values owing to the fact that chyle interferes with the protein precipitation.

Some Serologic Tests

Precipitin Reactions.—If the serum of an individual carrier of hydatid cysts is mixed with hydatid cystic fluid, in proper proportions, in twenty-four hours at room temperature a copious precipitate occurs. No alteration occurs in the mixture when negative sera are used. This precipitation is due to the presence in positive sera of a precipitin or antibody which, when in contact with its corresponding antigen, hydatid fluid, forms a copious precipitate.

Tests of Active Cross Immunity.—This is a biological test which is used for the classification and identification of trypanosoma and piroplasmas and of spirochetes which may morphologically be identical to other species in their same genera. A sheep is inoculated with a known strain of trypanosomes, or of either of the other two organisms, and develops the disease. This same animal is then inoculated with the trypanosome under study. If the animal again develops the disease, that is, from the new inoculation, then the organism used belongs to a different strain from the first. If the sheep does not acquire the infection from the second inoculation, then the organism in question is the same as that used for the first inoculation. In other words, if the strains are in reality identical both morphologically and biologically, then no disease develops from the second inoculation. Proof of infection is made by transferring the inoculation to another animal, since the parasites are too scarce to be found in the blood by means of a simple microscopic examination. This test is based on the active immunity which certain spirochetes, trypanosomes, and piroplasmoses confer on the host. However, lack of specificity detracts from the value of the test, as in syphilis and yaws.

Physical Alterations

The Ascoli Miostagmin Reaction.—Not only is biological testing of sera important in the indirect diagnosis of parasitism, but also the physical alterations observed in certain parasitoses. In certain parasitic diseases such as hydatidosis, ancylostomiasis, syphilis, etc., a decrease in the surface tension of the sera is noted, and consequently an increase in the number of drops in a given volume and at a given temperature. This is detected by the stalagmometer of Traube. A mixture of an antigen such as hydatid fluid and an antibody such as the serum from a hydatidosis patient produces a decrease in surface tension in the serum. Consequently the number of drops in such mixtures is greater than the number of drops in a mixture of the same antigen and normal serum. This reaction has been called *miostagmin* ("fewer drops").

Blood Sedimentation in Parasitoses.—The sedimentation test has been used in certain parasitoses, for instance by Giovani Souderi in Sicily, who noted an increase in blood sedimentation in ancylostomiasis, and by Bacigalupo in Buenos Aires who saw no change in sedimentation rate of the blood in six cases of necatoriasis. This writer believes that the increased sedimentation rate which he has seen at times in parasitic anemias is due to the anemia and not to the parasitosis. Our experiences with the anemias due to *Necator americanus* show that in this condition there is an accelerated sedimentation rate, which is retarded and becomes normal with the administration of iron in large doses. This therapy at the same time causes the anemic state to return to normal, with a lessening of the number of parasites as judged by the decreasing number of eggs in the stools after administration of iron.

This test can be recommended in parasitic diseases, especially in hookworm disease, in hepatic distomiasis, hepatic amebic abscess, hydatidosis, malaria.

CONCENTRATION OF STOOLS

Fülleborn Method

For the examination of *Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides,* and *Trichuris trichiura* eggs, a very good method is that proposed by Fülleborn, known as the "Hamburg Cover Glass Method." In this method, the infection and its severity can be determined somewhat by the number of eggs found.

One should always use the same magnification in looking for eggs so that the mind's eye will have the same picture of relative size. Use a No. 3 ocular with a Leitz No. 4 objective followed by Leitz No. 6, or the equivalents in other makes of microscopes. In order to find eggs in the urine, the urine must be centrifuged.

Use a special metal box devised by Fülleborn which has a depression in the center which holds about one gram of stool. After this depression is filled with stool, add a small amount of concentrated sodium chloride solution and mix thoroughly, adding enough sodium chloride solution to fill to the mark on the box which holds about 20 c.c. (The sodium chloride is made up in a large Florence flask half full of water. Add a large quantity of the salt so that it will be a supersaturated solution. Be sure to shake it at least five minutes before using.)

This gives a dilution of 1 to 20. It requires one hour for the Trichuris eggs to come to the surface. Place on top of this fluid three cover glasses; let them float, allowing the material to stand ten minutes; if looking for Trichuris, one hour. With wide-toothed forceps, pick off the cover glass without turning it and drop it on a slide without compression.

Determine the number of eggs present by a special ruled cover glass 18 millimeters square in size, which is exactly one-seventh of the entire upper surface of the saline solution. Multiply the result by seven; this will give the number of eggs in one gram of stool. If the egg quantity in one gram of stool is over 10,000, proceed as in counting red blood cells. Count ten different parts of each preparation, each one square millimeter in size. 3 × 10 will equal 30 counts. Each square represents an area of 18 × 18 or 324 square millimeters. Obtain the average counted in 30 squares and multiply by 324.

Telemann Method

There is another method, called the Telemann[1] method, which is suitable for all eggs, as well as the above varieties.

Take five pea-sized pieces of stool from five different parts on a glass rod. Mix with 50% hydrochloric acid and rub thoroughly. Add a small amount of ether, and shake. Place a fine mesh piece of copper gauze about five inches square inside a glass funnel. Let the fluid run through the copper gauze into a centrifuge tube. Centrifuge two or three minutes. You will then note an upper layer of ether, a lower layer of detritus; the eggs will be at the bottom. Pour off the supernatant fluid very carefully. Pick up the sediment with a pipette and examine on a glass slide.

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[1] Deutsche med. Wchnschr. 34: 1510, 1908.

Hunter Modifications of the Acid-Ether Sedimentation Technic of Telemann

These two methods were suggested by Hunter and associates.[2] Acknowledgment of these modifications came in a personal communication from Col. Hunter, to whom we express our gratitude. The modifications devised by him are called the AMS III method and the 406 MGL method. Col. Hunter has used both these methods routinely and has found the MGL superior to the zinc sulphate technic for protozoa and worms. The AMS III is better than the MGL for the recovery of helminth eggs and larvae. "It was designed for schistosomiasis, but we have used it on over 20,000 stools here in the Far East where it has been run in parallel with the MGL and have found it to be excellent all around technique for eggs."

THE AMS III METHOD

Reagents.—

Hydrochloric Acid-Sodium Sulphate Mixture.—

Prepare hydrochloric acid of a specific gravity of 1.089 by adding 45 c.c. of concentrated 37% hydrochloric acid to 55 c.c. of water.

Prepare sodium sulphate of a specific gravity of 1.080 by dissolving 9.6 gm. of anhydrous sodium sulphate in 100 c.c. of water.

Add equal parts of the hydrochloric acid and sodium sulphate solutions. The final mixture has a specific gravity of 1.080. Each solution must be adjusted to the proper specific gravity, if needed, before mixing the final mixture.

To obtain the best possible results, it is advisable to dry the sodium sulphate before using. Dry it in a hot-air oven for 2 hours at 130° C. and cool in a desiccator. Weigh immediately upon removing from the desiccator.

Triton NE (Triton X30).—

This is a wetting agent.[3]

Ether.—

Technic.—

Partial comminution of the entire stool with an appropriate amount of water can be accomplished in the stool box. A suitable amount of water will make it possible to recover 10 to 12 c.c. of strained emulsion, which when centrifuged will yield 1 to 1.5 c.c. of fecal sediment.

Strain through 2 layers of gauze moistened with hydrochloric acid-sodium sulphate mixture into a 15 c.c. centrifuge tube.

Wash 3 times by brief centrifugation, 1½ to 2 minutes at 1,700 to 2,100 r.p.m., decanting the supernatant fluid each time and mixing the sediment with fresh acid-sodium sulphate mixture.

After decanting, add 5 c.c. of hydrochloric acid-sodium sulphate plus 3 drops of Triton NE plus 5 c.c. of refrigerated ether.

Shake for 30 seconds and centrifuge one minute.

Remove the tube, break the ring at the interface with an applicator, and decant. Swab the tube down to the sediment with a cotton swab, being careful not to touch the sediment.

Add tap water to the 0.4 c.c. mark, mix the sediment, and pour as much as can be read onto a slide.

Cover with a 24 by 40 mm. cover glass.

An applicator stick may be used to draw the few drops of sediment to the lip of the tube. It is especially useful in controlling the amount of sediment that escapes onto the slide.

Examine under the low power of the microscope. There should be a minimum of debris and the eggs should stand out clearly. Some of the mature eggs may exhibit viable miracidia.

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[2] Bull. U.S. Army Med. Dept., Feb., 1943.

[3] Triton NE may be obtained from Rohm and Haas Co., Philadelphia, Pa.

THE 406 MGL METHOD

Partial comminution of the entire stool, with an appropriate amount of water, can be accomplished in the stool box. A suitable amount of water will make it possible to recover 10 to 12 c.c. of strained emulsion, which when centrifuged will yield 0.5 to 1 c.c. of fecal sediment.

Strain through 2 layers of gauze. Collect the emulsion in a 15 c.c. centrifuge tube.

Centrifuge and decant the supernatant fluid. Additional washings are advantageous.

Mix the remaining fecal sediment thoroughly with 10 c.c. of 10% Formalin. Allow 10 to 30 minutes for fixation. This reduces distortion of protozoan cysts.

Add about 3 c.c. of ether to the formolized specimen.

Stopper the tube and shake vigorously.

Centrifuge at relatively low speed for about 2 minutes.

Completely decant the ether, superficial debris, and Formalin, using an applicator stick to free the superficial debris from the centrifuge tube.

Mix the sediment remaining in the centrifuge tube thoroughly with the fluid that drains back from the tube wall, and pour onto a glass slide. Avoid an excess.

Place a small drop of 2% iodine solution near the top of the sediment, and mix by using the edge of a cover slip.

Push the edge of the cover slip into the drop, allowing the fluid to run under the cover slip and at the same time pushing the coarse debris aside. This step is critical in obtaining a suitable microscopic preparation.

Loughlin and Stoll Modification of the Telemann Technic

Stoll and Loughlin[1] described a modification of the Telemann method. This modified technic consistently demonstrated infertile Ascaris eggs. It also possesses a similar degree of effectiveness for hookworm, fertile Ascaris, Trichuris, *Hymenolepis nana,* and *Schistosoma japonicum* eggs. This method does not inherently preclude the demonstration of eggs which cannot float in solutions of high speed gravity, nor does it cause such loss of eggs from the material to be examined as occurs in the Telemann procedure.

Measure 4 c.c. (or grams) of feces into a dilution counting flask which has been filled to the 56 c.c. mark with water.

Add several glass beads (6 mm.) and after giving the preparation an initial shaking, set aside for several hours or overnight (preferably refrigerate); complete the comminution of the feces by vigorous shaking, so that all the eggs are free.

Shake to produce a thorough distribution of the eggs in the fecal suspension and transfer immediately 1.5 c.c. of the suspension to a 15 c.c. pointed centrifuge tube.

Add 3.5 c.c. of 20% hydrochloric acid,[2] put a rubber stopper in the tube, and shake for one minute; allow to stand for two minutes.

Add 5 c.c. of a freshly prepared mixture of equal parts of ether and xylol and again shake for one minute.

Centrifuge at 1800 to 2000 r.p.m. for two minutes and allow the centrifuge to come to a stop gradually without interference.

Separate the semifloating coagulum from the walls of the tube with a thin wooden applicator.

Decant quickly, permitting the sediment in the bottom of the tube to remain undisturbed. Then, while holding the tube almost horizontally, clean any adhering coagulum from the inside of the tube with an applicator covered with gauze.

Add one drop of a N/10 solution of sodium hydroxide to the sediment, mix thoroughly with a capillary pipette, transfer the entire suspension to a glass slide and place a cover glass on it. If the density or amount of residue warrants it, make two drops of the material and cover each with a coverslip in order to secure a preparation which will allow the eggs to be seen easily. Stoll and Loughlin prefer a 1½ by 3 inch slide and a 22 by 30 mm. or 25 mm. square coverslip.

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[1] Am. J. Trop. Med. 26: 517, 1946.

[2] 20 c.c. of concentrated hydrochloric acid diluted to 100 c.c. with distilled water. The net concentration of HCl in the centrifuge tube is reduced to 14%.

Examine the whole preparation for eggs. Those found are from 0.1 gram of the original fecal specimen.

For Egg Counting.—At will, or whenever eggs are demonstrated, the displacement flask preparation may be used for routine dilution egg counting. In order to secure an approximate decinormal sodium hydroxide suspension without appreciably altering the original dilution, one hundredth the volume of 10 N sodium hydroxide[3] is added. Thus, if 1.5 c.c. have been removed from the 60 c.c. suspension, 0.58 c.c. of the 10 N sodium hydroxide is added. One can proceed at once with dilution counting, although it is best to allow the flask with its contents to stand for an hour after shaking.

The use of hydrochloric acid of specific gravity 1.030 in this method provides satisfactory acid disintegration of the feces and permits eggs of all species to be easily sedimented by centrifugation. The addition of the xylol diminishes the adhesive properties of the coagulum of the fecal debris and prevents foaming common to the use of ether alone.

Willis Method of Concentration of Stools

The method is based on the property of eggs of certain worms to float to the surface of a concentrated salt solution, and to adhere to a glass surface.

In a glass cylinder, no larger than 2.5 cm. in diameter, carefully and thoroughly emulsify no less than one gram of fecal material with saturated solution of sodium chloride. The salt solution should be freshly prepared. Add more of the salt solution, and continue the emulsification until the container is filled to the top and a meniscus forms. Wait at least five minutes for the eggs to float to the meniscus. Then carefully place a glass slide over the surface of the solution and allow it to remain for 10 to 30 minutes. Take care to admit no bubbles.

Carefully remove the slide and turn it upside down without spilling the liquid which contains the eggs. Cover with a thin cover glass and examine under the microscope.

This is an excellent general concentration method, but it is of doubtful value in examining for eggs of the trematodes.

Acetic Acid and Ether Concentration Method for the Examination of Feces*

In a comparative study of various methods of concentration for the examination of feces the following method has been found to give greater amount of concentration, with the added advantage of eliminating most of the detritus in the material concentrated. The technic is practical and very simple. It requires only a few minutes to complete the work and make the examination. The method is based on the addition of a 5 per cent solution of acetic acid to diluted fecal matter. The purpose of this acid is to dissolve the carbonaceous matter and to clear the mucous material which may be present; it may also hydrolyze the fatty substances to some extent. Ether, which is added, dissolves the excess fatty substances and, by infiltrating the suspended particles, renders them lighter, facilitating their separation to the top of the mixture and the settlement of eggs, cysts, cells, and other heavier particles by centrifugalization.

Soften about 1 or 2 gm. of fecal material, if necessary, and suspend in about 10 c.c. of 5% solution of acetic acid in a test tube.

Shake thoroughly for a few seconds.

Allow to stand for about a half minute to permit the heavy particles to settle to the bottom of the tube.

Filter through a single or double layer of gauze and collect the filtrate in an ordinary test tube. The tube should be about one-third full.

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*Am. J. Trop. Med. 8: No. 1, 1928.

[3] Prepared by dissolving 40 grams of sodium hydroxide sticks in distilled water q.s. to 100 c.c. Note that this concentration is caustic.

To the filtrate in the tube, add an equal volume of ether, and stopper the mouth of the tube tightly with a rubber stopper.

Shake vigorously until a thorough mixture is obtained. This can be judged by an emulsion-like appearance of the material. This shaking is usually complete in from 15 to 30 seconds.

Immediately centrifuge the tube for two, three, or five minutes. This procedure separates the material into four well-marked layers. The topmost layer is the ethereal extract. Immediately below this is a plug of detritus, which is made up of coarser particles, proteins, and soap. The acid solution is below this plug, and a small amount of sediment is at the bottom of the tube.

The three upper layers, the ethereal extract, detritus plug, and acid layer, can be easily removed by a gentle jerk of the tube, leaving the sediment adhering to the bottom of the tube. If this is done carefully, the sediment will remain in contact with the tube.

Collect the sediment with a capillary pipette and place on a slide.

Drop a cover glass over the sediment and examine under the microscope. The material may be fixed and stained.

This method gives a much higher degree of concentration than do many other methods. It facilitates the rapid detection of eggs, protozoa cysts, and cellular elements by its high degree of concentration and by the clearing of detritus from the preparation. It can be used by any laboratory technician with ease and confidence.

Formol-Ether Stool Concentration, Ritchie[1]

Mix stools with about 10 volumes of tap water.
Strain through gauze into 15 c.c. centrifuge tubes.
Centrifuge the strained emulsions.
Wash the sediments with tap water.
Repeat the centrifuging and the washing.
Decant the supernatant fluid.
Add 10 c.c. of 7.5% Formalin to the sediment.
Let stand for 10 to 30 minutes.
Add about 3 c.c. of ether.
Stopper the tubes and shake vigorously.
Centrifuge at low speed for about 2 minutes.
Free the superficial debris from the tube wall with an applicator stick.
Then decant all the supernatant mixture.
Examine the sediment under a microscope.
Add 2% iodine and examine again.

This method gives favorable results with protozoa and helminths. It is possible to add the Formalin to the stools at the time of their collection and carry out the centrifuging and flotation later in the laboratory. In this case, the Formalin serves as a preservative for the parasites and their eggs.

Zinc Flotation Method of Concentration of Eggs in Parasitologic Examinations

A preliminary communication by Faust, D'Antoni, Odom, Miller, Peres, Sawitz, Thomen, Tobie, and Walker[2] gives credit for the centrifugal flotation technic and counts of parasitic eggs to John Tobie. This flotation method ultimately adapted was as follows:

Mix a small amount of feces (about the size of a pecan) with about 10 parts of water.

Strain 10 c.c. of the suspension through a *wet* cheesecloth in a funnel into a Wassermann test tube.

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[1] Bull. U.S. Army Med. Dept. **8**: 326, 1948.

[2] Am. J. Trop. Med. **18**: 169, 1938.

Centrifuge for 45 seconds to one minute at top speed.

Pour off the supernatant fluid.

Add 2 or 3 c.c. of water and break up the sediment by shaking or tapping.

Add more water to fill the tube.

Centrifuge and repeat this washing and centrifuging until the supernatant fluid is clear. Pour off the last supernatant fluid.

Add 3 to 4 c.c. of a solution of zinc sulphate, specific gravity of 1.180.

This is a 33 per cent solution of granular zinc sulphate in water (386 gm. of granular zinc sulphate to 1 liter of distilled water). Shake. The material should be up to within one-half inch of the rim of the tube.

Centrifuge for one minute at top speed.

Remove the surface film by means of a platinum loop. Place on a slide and add 1 drop of D'Antoni's iodine stain. Agitate by hand and mount. Examine.

It has been found by this method that all the eggs, even operculated eggs, will rise to the top.

Ronald-Elsdon Dew* in discussing this method advises that specimens containing much mucus are not suitable for this technic, as the mucus floats *in toto,* and there is little gain in concentration. He recorded the findings of 1,539 specimens in which this technic was compared with the direct fecal film. The over-all gain by the use of this technic was 179 per cent. Some eggs do not float well, for instance Taenia and *Schistosoma mansoni.* They can often be found in the sediment of the final "spin."

Baroody's Modification of the Faust Method for Concentration of Cysts and Eggs

Faust's method for the detection of cysts and eggs embodies the use of zinc sulphate method for flotation purposes. Baroody[1] called attention to the fact that the routine method of Faust does not call for enough stool sample to permit finding cysts of *Endamoeba histolytica* when they are present in small amounts. He therefore modified the Faust method by taking a larger quantity of stool and in this way succeeded in finding cysts where he had failed to do so with the original Faust method. His modified technic is a combination of Baroody and Most's water centrifugal sedimentation method,[2] page 1811.

Technic.—

Place about 10 gm. of feces in a 125 c.c. Erlenmeyer flask containing about 75 c.c. of warm water (about 40° C.); stopper the flask and shake until emulsified.

Strain through 1 layer of wet gauze into a 50 c.c. centrifuge tube (teated bottom).

Centrifuge for one minute at 2500 r.p.m. and pour off supernatant fluid. Add warm water to the sediment from a regulated faucet (force of the water will disperse the fecal particles and cleanse them) and centrifuge again at the same time and speed. Decant supernatant fluid; repeat one more washing and centrifugation; again decant supernatant fluid.

Add about 10 c.c. of warm water and agitate sediment by shaking; then pour quickly all of contents into a 15 c.c. centrifuge tube. Centrifuge for one minute at 2500 r.p.m. and decant supernatant fluid.

Add about 10 c.c. of zinc sulphate solution (33 per cent, specific gravity 1.180) to the sediment and stir with applicator sticks. Add zinc sulphate nearly to top of tube. Centrifuge for one minute at 2500 r.p.m. Eggs and cysts will float to the surface.

Take several loopfuls (bacteriologic loop) from surface and place on slide; add 1 drop of D'Antoni's or Lugol's iodine and cover with a cover slip. Examine for eggs of helminths and cysts of protozoa under low-power objective of microscope.

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*Tr. Roy. Soc. Hyg. 41: 213, 1947.

[1] J. Lab. & Clin. Med. 31: 1372, 1946.

[2] J. Lab. & Clin. Med. 31: 815, 1946.

It will be noted that in this method a much larger sample of feces is utilized, thereby increasing possibility of detection in lightly infested cases or when cysts and eggs are rare in the stool. Cysts may exist in "packets" and can be missed from a remote small section. The use of warm water (about 40° C.) has advantages over the use of regular or so-called cold water. The use of warm water eliminates a high percentage of the scum which has been observed when cold water was used. After the water is added the scum rises to the surface within one minute, remains floating after the centrifugation, and eventually is discarded with the supernatant fluid. Each additional washing brings up less scum until it is practically all discarded. When the zinc sulphate is added, the surface of the liquid is fairly clean and makes a good field for microscopic examination. The initial use of the large 50 c.c. test tube instead of the smaller test tube is important because it is necessary to receive a large sample from the original 10 gm. of stool. With the larger tube the proportion of sediment to the size of the tube decreases the number of washings and centrifugations in arriving at a clear supernatant fluid; three or four washings usually give this desired result and a clean zinc sulphate preparation.

This method is particularly useful in looking for the cysts of *Endamoeba histolytica* as well as Strongyloides larvae. It is of particular value when cysts, eggs, and larvae are rare in the fecal specimens.

Baroody's method is recommended because any acid-ether technic will destroy immature *Schistosoma japonicum* eggs, thereby offering at least a partial explanation of the many negative results encountered.

Further, in the laboratory diagnosis of *Clonorchis sinensis,* the diagnosis was made by the Baroody method when zinc sulphate and brine flotation methods were negative. Acid-ether technic was also positive.

Water Centrifugal Sedimentation Method for Demonstration of Schistosoma Japonicum Eggs

Method of Baroody and Most[1]

Place 10 to 15 gm. of the feces in a 125 c.c. Erlenmeyer flask containing about 100 c.c. of lukewarm tap water. Insert a No. 4 rubber stopper and shake vigorously for 1 or 2 minutes or until the particles are in fine suspension.

Strain the emulsified feces through two layers of wet gauze into a 50 c.c. centrifuge tube with teated bottom (tube dimensions: height, 4¾ inches; diameter, 1 inch).

Centrifuge at 1500 r.p.m. for 30 seconds. Apply the brake of the centrifuge gradually.

Decant the supernatant fluid and add tap water at about 40° C. to the 50 c.c. mark on the tube. Insert a rubber stopper and shake several times until the sediment is finely dispersed. The warm water causes a scum to rise to the surface. This is desirable.

Repeat the centrifuging and washing until the supernatant fluid is clear; three washings and three centrifugations are usually sufficient.

After the last washing and centrifugation, pour off the clear supernatant fluid; the packed sediment remains. Gently tap the finger against the side of the tube to disperse the packed sediment which will settle to the bottom of the tube by gravity within a minute.

With a rubber-bulbed pipette place 4 drops of mixed sediment on a glass slide and cover with a large cover glass size 22 by 50 mm. Make at least two slides.

Examine under the low power objective of the microscope for eggs of Schistosoma.

Schistosoma japonicum eggs are oval, 70 to 100 by 50 to 60 microns, may or may not have a rudimentary spine, and in blue-white light they appear greenish orange. *Schistosoma mansoni* eggs are elongated, 114 to 175 by 45 to 68 microns, and have large lateral spines. In both species, if the eggs are visible, the miracidia will exhibit movements within the eggshells, and the activity of the flame cells may be seen under higher magnification.

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[1] J. Lab. & Clin. Med 31: 815, 1946.

If the slides are negative for schistosoma eggs, add about 10 drops of water to the remaining sediment in the tube, leave overnight in a warm place, and examine the following day for miracidia. The entire contents of the tube can be placed on a large slide and examined under a dissecting microscope. This phase of the examination requires less than 2 minutes.

Excluding the last procedure, not more than 5 minutes are required for the examination of each slide, and not more than 15 to 20 minutes are required for the entire method.

Other eggs, cysts of protozoa, and larvae of helminths may also be found and identified. For protozoan cysts, add a drop of Lugol's or D'Antoni's iodine to the slide.

Preserved concentrated specimens can be made quickly by adding 10 per cent Formalin to the sediment in the tube at the end of the fresh examination.

This water centrifugal sedimentation method is a simple rapid one, requiring no chemical reagents, and only a minimum amount of standard laboratory equipment. The method is to be recommended especially in treated cases particularly when the other concentration methods have been tried and have proved unsuccessful.

ANAL SWABS

Special Procedure for Recovering Eggs in Oxyuriasis

It is a well-known fact that the eggs of *Enterobius vermicularis* are not deposited in the intestinal canal, as are those of hookworms, ascarids, and many other worms, but are expelled by the gravid female after it has migrated out of the anus and onto the perianal region. For this reason, the examination of samples of stools for pinworm eggs is a haphazard and unreliable method of diagnosing pinworm infestation. The stools of the vast majority of patients with pinworms will not reveal pinworm eggs, and although the occasional positive findings are significant, the negative findings afford no information as to the existence or absence of infestation. In an interesting contribution on this subject, Wright and Cram* state that in 102 examinations of the stools of persons infested with pinworms, they found pinworm eggs in only fourteen cases, and most of the patients were persons heavily infested with worms, as determined by subsequent treatment. In other words, the results were misleading in about seven of every eight cases.

In order, therefore, to make a reliable diagnosis of this disease, the anal swab, as suggested by Hall,[1] or any of the other swab methods given here should be used. Various types of swabs have been used by various workers, notably cotton, chamois, and rayon swabs. Celluloid swabs, depending on a scraping action instead of an entangling or enmeshing action in collecting eggs, have been given consideration by various authors. Serbinow and Schulmann[2] used a pointed, spatula-shaped match moistened with 0.5 to 1 per cent solution of sodium hydroxide, which was passed lightly over the anal folds; the material was examined in sodium hydroxide or water. Bogojawlewski and Lewitzki in 1929 used an ordinary match moistened with 1 per cent sodium hydroxide and examined the scraping in 1 per cent sodium hydroxide; when the initial scraping was negative, the edge of the slide, as well as the match, was used in scraping.

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*Am. J. Dis. Child. 54: 1276, 1937.

[1] Am. J. Trop. Med. 17: 445, 1937.

The NIH Swab (Hall).—

Hall first suggested the use of a metal scraper but later worked out a very satisfactory cellophane swab. This is made by taking a piece of transparent cellophane about 3 cm. square, folding it across the middle to form two equal rectangles, placing a glass rod about 8 to 10 cm. long by 4 mm. wide, with rounded ends, between the halves of the folded square and at right angles to the fold about one-third of the distance from one end of the fold, folding down the short end diagonally and wrapping it about the short end of the rod, and fastening the folded cellophane to the rod with a small rubber band. The rod was first pushed through a perforated rubber cork of a size suitable for corking a test tube. This combination of cellophane, glass rod, rubber cork, and test tube constituted a simple setup which met several requirements for a satisfactory swab for large-scale operations. The swab was used dry, and pinworm eggs adhered to it or were scraped into the folds over the tip. Thus it acted both as a swab and a scraper. To make the examination for eggs, the cellophane was pushed forward over the tip of the glass rod for a short distance, and the cellophane tip which had been used for swabbing was cut off beyond the rod tip with a pair of fine scissors, the tip being held at the time in a mixture of decinormal sodium hydroxide and stain; the cellophane tip was then flattened out with the rod and a pair of forceps, one or two drops of additional sodium hydroxide were put on the cellophane, and the preparation was covered with a cover glass. The eggs adhering to the cellophane or released under it were readily found by microscopic examination through the thin transparent cellophane, and no separation of the eggs from the contact part of the swab by any form of manipulation was needed. There are two types of cellophane, the plain transparent and the moisture-proof transparent. The moisture-proof cellophane flattens somewhat less readily on removal from a swab prepared or used several days or weeks previously than does the plain transparent cellophane. Hall suggests the use of the moisture-proof cellophane. The cellophane flattens a bit more readily if the slide preparation is warmed. The new type of swab suggested by Hall is called the NIH swab.

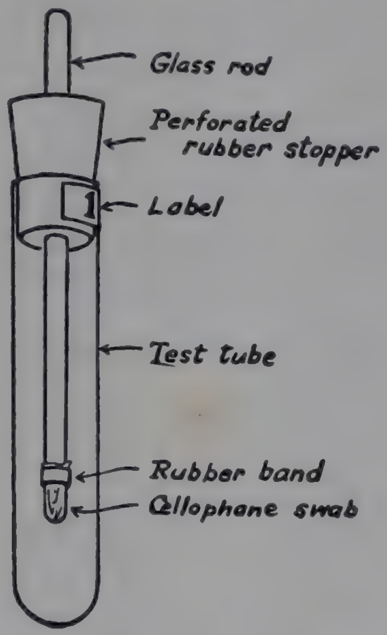

Fig. 447.—The NIH swab (Hall).

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[2] Arch. f Schiffs- u. Tropen-Hyg. 31: 482, 1927.

After the cellophane is removed, the rod and cork are returned to the test tube and the forceps are flamed. The test tube setup should be sterilized, preferably by dry heat, in order to kill and distort any eggs present and so to guard against infection or contamination when the cellophane is replaced and the swab used again.

The use of decinormal sodium hydroxide and a stain in the examination of various types of swabs, including the cellophane swab, has been found advantageous since the solution tends to saponify fats in the feces and thus to set free the worm eggs in fecal material.

A warning note on the use of these cellophane NIH swabs has been issued by Reardon.* She states that artefacts which simulate eggs of *Enterobius vermicularis* are frequently found in this cellophane. These artefacts are not superficial but are embedded in the cellophane film and cannot be dislodged by surface scratching. They show a hyaline outline, ovoid, sometimes rounded, resembling very closely a pinworm egg. Size variation is great, but usually falls within the range of the pinworm egg so that size and appearance constitute potential sources of error in diagnosis of oxyuriasis.

In addition to the NIH swab just described, the following methods were summarized by Watson and MacKeith.[1]

The **Graham swab** introduced by Graham[2] in which adhesive tape (Scotch tape) is applied to the perianal region with the finger or a glass rod, the tape being subsequently flattened, adhesive surface downward, on to a slide for microscopic examination.

The **TP swab** introduced by Watson and MacKeith (1946). This device employs tissue-paper or toilet-paper in place of ordinary cellophane, the paper being applied either with the finger or by using a glass rod and rubber band. It is afterward rendered transparent by placing it, applied surface upward, in a drop of clearing agent such as oil of wintergreen. Another drop of clearing agent is added and the preparation covered with a cover glass. The eggs do not clear for some considerable time and are therefore readily visible against the transparent background.

The **glass pestle method** devised by Schüffner and Swellengrebel[3] which consists of a piece of thick-walled glass tubing of about 1 cm. diameter having a globe blown at one end, half the surface of which is rough ground. The moistened globe is applied to the perianal skin with a rotary motion and then dipped in a drop of water on a slide; the drop may be examined immediately or allowed to dry and examined later after clearing with a drop of cedarwood oil.

The **direct slide method** introduced by Petersen and Fahey.[4] This merely consists of the application of an ordinary microscope slide to the anal mucosa and the mucocutaneous junction, the moisture of the anal margin being sufficient to make any eggs present adhere to the slide. The slide may be examined direct or after addition of decinormal sodium hydroxide solution.

The **camel's-hair brush method** introduced by Watson and MacKeith (1946). This is an adaptation of the method used by Nolan and Reardon[5] for collecting household dust for examination for threadworm eggs. The brush is moistened with water and applied with a stroking motion to the anal folds and perianal skin. For examination it is gently dipped into a few drops of decinormal sodium hydroxide solution on a deep well-slide.

The **petrolatum cloth method** devised by Hellsten[6] in which the perianal region is wiped with a petrolatum cloth, the material obtained shaken up with a mixture of water and ether, centrifuged, and the suspension examined for eggs.

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*Am. J. Trop. Med. 18: 427, 1938.

[1] Tr. Roy. Soc. Trop. Med. & Hyg. 40: 376, 1947.

[2] Am. J. Trop. Med. 21: 159, 1941.

[3] Zentralbl. f. Bakt. 151: 71, 1943.

[4] J. Lab. & Clin. Med. 30: 259, 1945.

[5] J. Parasitol. 25: 173, 1939.

[6] Nord. med. tidskr. 6: 1358, 1933.

METHODS OF PRESERVATION OF GROSS SPECIMENS OF PARASITIC EGGS OR EMBRYOS

Stools containing parasitic eggs or embryos are best preserved by adding 40 per cent Formalin to a thin watery suspension of the material, so that the latter contains about 2 per cent of Formalin. The majority of eggs are quite well preserved in this manner, although some of the thin-shelled eggs, such as *Hymenolepis nana,* show considerable distortion. Specimens of feces containing the rhabditiform embryos of *Strongyloides stercoralis* may be kept for several years; the structure may be lacking in some of the embryos, but may remain characteristic in others. It may be of interest to note that the Formalin does not arrest the development of the eggs of *Ascaris lumbricoides.* Morris[1] reports one specimen of feces which, after four years' preservation in Formalin, still showed living embryos in Ascaris eggs.

Adult specimens may also be preserved in 2 per cent Formalin.

Permanent Preparations of Flatworms

These may be prepared in several ways:

1. Method of Mink and Ebeling[2]

The fecal material is mixed with physiologic salt solution heated to about the body temperature (37° to 40° C.). The worms move about, and the smaller, such as *Hymenolepis nana,* are the more readily seen. With forceps the parasites are transferred to a second dish of clear salt solution, in which they become free of mucus and feces. They are now transferred to one of three solutions for fixation: (1) Alcohol, 50 to 70 per cent, with or without glycerin; (2) Zenker's solution (which consists of bichloride of mercury, 5.0 gm.; potassium bichromate, 2.5 gm.; sodium sulphate, 1.0 gm.; distilled water to 1,000 c.c.), or (3) a 2 per cent Formalin solution; in any of these the material remains 14 to 16 hours. Zenker's fluid causes considerable shrinking and a yellowish discoloration. Formalin is best, since the natural color of the parasite is preserved fairly well with little or no shrinkage. The fixative should be allowed to act not more than fifteen hours, when the parasites are transferred to the following medium:

Syrup (glucose, 48 parts, water, 52 parts)	1,000.0 c.c.
Methyl alcohol	200.0 c.c.
Glycerin	100.0 c.c.
Camphor, q.s. (to keep sterile).	

The specimens may be left in this solution indefinitely, though they are usually sufficiently cleared in four to five hours. The material is now placed on a slide in glycerin jelly. After the latter has hardened (twenty-four hours or more), the cover glass is sealed with cement.

2. Boggs Method

The worm is washed free of feces, and is left in water or salt solution until it dies, so that it may be fixed while relaxed. It is then placed in a solution of 20 per cent glycerin in 80 per cent alcohol, which both fixes and clears the specimen. It is kept in this fluid in a partially covered dish until the alcohol is entirely evaporated. The specimen is then clear. It is transferred to a glass slide, and the excess of glycerin is removed by blotting paper. Glycerin jelly is then placed on the specimen, which is covered with a cover glass. In twenty-four to forty-eight hours, after the jelly has solidified, the preparation is sealed with microscope cement. To prevent curling of the specimen, it may be spread on a piece of heavy filter paper before immersing it in the glycerin-alcohol solution; it may be necessary to put a light weight on the cover slip until the jelly hardens.

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[1] U.S. Naval Med. Bull. 3: 267, 1909.

[2] Bull. Johns Hopkins Hosp. 22: 299, 1911.

Glycerin jelly is prepared as follows:

Gelatin (gold mark)	14.0 gm.
Distilled water (boiling)	120.0 c.c.
Dissolve in the hot water and add:	
Glycerin	120.0 c.c.

Cool to 50° C. and add the whites of two eggs. Heat gently without stirring. Strain the mixture through a fine-meshed wire sieve, and filter through cotton while still warm. Add water to make the volume 240 c.c. and 1 c.c. of pure carbolic acid as a preservative. The jelly solidifies on cooling. For use, melt it by immersing the flask containing it in hot water.

3. Creosote Method

The material is placed in 70 per cent alcohol, and then in 95 per cent alcohol, for fifteen to thirty minutes. It is then transferred to Beechwood creosote, in which it remains until the tissue is cleared. The time required varies with the size of the specimen and with the degree to which water was withdrawn by the alcohol. Finally, the specimen is mounted in balsam.

4. Permanent Mounts of Helminth Eggs in Aqueous Preserving Fluids

It is often desirable to prepare permanent mounts of helminth eggs by a simple technic which will eliminate the shrinkage and distortion that frequently occur in the longer process of dehydration and subsequent mounting in gum damar. In the following technic suggested by Walker,[1] the eggs are mounted permanently on the slide in 4 per cent formaldehyde:

1. Place the slide on a turntable and ring a cell with Murrayite.

2. Fill the cell immediately with the preserving fluid (4 per cent formaldehyde) containing the eggs. An excess of the preserving fluid should be placed in the cell to prevent trapping air bubbles.

3. Place a circular cover glass in position immediately. With gentle pressure of the index fingers rotate the cover glass through an arc of approximately 30 degrees, thus pressing the edge into the soft cement. Usually the cover glass will adhere, even though the rim may have become wet with the overflowing preserving fluid.

4. Allow the slide to dry overnight and then re-ring it to insure a more permanent seal.

5. Twenty-four hours later the slide can be rinsed in running water, dried with a cloth, and labeled.

Similar permanent mounts of adults and larvae of *Trichinella spiralis* have been made by preserving them in an aqueous solution of 4 per cent formaldehyde and 30 per cent glycerin.

Murrayite adheres readily to the cover glass, even though the cover glass has been moistened previously with the preserving fluid. This is a distinct advantage, since in the use of other ringing cements it is necessary that the edges of the cover glass be absolutely dry. Murrayite is a spirit-proof cement used in sealing museum jars and microscopic fluid mounts. It was invented by Dr. C. Hay Murray, of Liverpool, and is sold by one or more American Biological supply houses.

5. Lacto-Phenol

Lacto-Phenol.—

Glycerin	2 parts
Lactic acid	1 part
Phenol	1 part
Water	1 part

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[1] Science, June 17, 1938.

Technic.—Place whole worms in lacto-phenol solution and allow them to remain in the solution indefinitely. This solution renders the worms transparent and preserves them. It is highly recommended. *Caution.*—If the lacto-phenol solution is improperly made, it will digest the worm instead of preserving it.

6. Gum Acacia-Chloral Hydrate Medium, Ratcliffe*

Prepare a series of dilutions of gum-chloral in 10 per cent Formalin beginning with a 10 per cent solution and increasing concentrations by 2 per cent for each subsequent step, or make up each dilution as it is needed.

Gum-chloral is prepared as follows:

Distilled water	50 c.c.
Gum arabic ACS (must be clean)	40 gm.
Glycerin	20 c.c.
Chloral hydrate	50 gm.
Acetic acid	8 c.c.

Dissolve the gum arabic in the water cold, then dissolve the chloral hydrate with gentle heat, add the glycerin and acetic acid.

Concentrate fecal suspensions that have been thoroughly fixed in neutral Formalin until each drop of fluid contains 10 to 12 eggs. Each cubic centimeter of this will yield about 10 slides.

Pipette 5 c.c. of the concentrated fecal suspensions into 15 c.c. vaccine bottles. Add an equal quantity of 10 per cent gum-chloral-Formalin, tilting the bottles to allow this fluid to run in along the lower side. In all dilutions of the series, gum-chloral is heavier than any fecal suspension tested and will occupy the lower half of the column of fluid. No not shake the bottles or stir the contents.

Cap the bottles and leave them at room temperature or in an incubator at 37° C. until the fecal material has completely settled. Then remove the clear fluid above the feces with a fine-pointed pipette attached to a vacuum pump, and add an equal volume of the next higher concentration of gum-chloral solution.

Continue this process until the eggs are suspended in full-strength gum-chloral medium. When the last sedimentation is complete and excess fluid removed, mix the contents of each bottle thoroughly before starting to prepare mounts.

Use clean slides and cover glasses. Circular cover glasses 12 to 15 mm. in diameter give better mounts than larger sizes. Squares are unsatisfactory for most specimens. Mounts are prepared by two persons working as a team; one transfers drops of the mixture to the slides, the other adds the cover glasses. With some practice one will come to judge the size of the drop which will spread completely under the cover glass without excess. For transferring the material to slides, a heavy platinum loop about 4 mm. in diameter is superior to a pipette. The loop delivers a drop of about the correct size with few air bubbles. Cover glasses must be applied quickly to the mounts. Otherwise the medium hardens and spreads poorly.

Dry mounts in a horizontal position in an incubator at room temperature. Any air bubbles under the cover glass usually are extruded during drying. Thereafter, slides may be treated as if prepared with balsam or Clarite. The medium is readily soluble in water, however.

This schedule requires several months for completion, but actually the time given to the specimens is only the matter of minutes for each change of solution. Results amply compensate for the effort. By this method Ratcliffe has prepared mounts of the eggs of *Schistosoma mansoni, Clonorchis sinensis, Fasciola hepatica, Diphyllobothrium latum, Hymenolepis nana, Taenia saginata, Ascaris lumbricoides, Trichuris trichiura* and *Enterobius vermicularis.* In these slides small numbers of the eggs of some species are distorted, but the majority remain intact and are seen in greater detail than in temporary mounts of Formalin-fixed feces. The only failure thus far has been with hookworm eggs, all of which became badly distorted.

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*Science 99: 2576, 1944.

Adult *Necator americanus* and other small nematodes, mites, ticks, lice, and the larvae and pupae of flies are easily infiltrated and mounted by this method. These organisms remain soft and are easily arranged on slides. When mounted under small round cover glasses, supports do not seem to be necessary.

PVA Medium, Morrison*

Polyvinyl alcohol 51-05 (Dupont Elvanol)	35 gm.
Distilled water	80 c.c.

Blend with a stirrer at 80° C. Then heat on a water bath at 95° C., just below boiling, until solution is effected.

Add

Chloral hydrate	25 gm.
Lactic acid	15 gm.

After mounting the specimen, dry at approximately 40° C. for a few days and then ring the cover glasses with a varnish after the slides are made.

PRESERVING FLUIDS

When a parasitologic examination cannot be carried out immediately, the stool is mixed with about 10 times its volume of 10 per cent Formalin. Formol-fixed material, however, is not very suitable for detailed protozoologic studies. Schaudinn's method is preferred if it is possible to carry it out with the facilities available. This fixative is also indispensable for the preparation of permanent hematoxylin-stained slides. Recently Schaudinn's fluid with the addition of polyvinyl alcohol (PVA) has been recommended (see page 1822).

For the latter purpose several slides, preferably with frosted edges, are prepared. Write the name and number of the patient with graphite pencil on the frosted glass. If the stool is hard, drop some saline on the farther end of the slides. For liquid stools, use a thin coat of egg albumin.

Mix the sample with the saline or spread over the egg albumin with the aid of a wooden applicator. Drop the slides immediately into a modified Schaudinn solution (page 1822).

Keep the fixative in a Koplin jar. Put the slides into it back to back. When 2 slides are made from each stool specimen, the material from as many as 5 stools can be fixed in 1 jar.

Keep the slides in the fixative for at least 8 hours, but not longer than 48 hours. They can then be mailed or sent by messenger to another laboratory for staining, if this is not carried out locally.

RECOMMENDED PROCEDURE FOR THE DETECTION OF INTESTINAL PROTOZOA

Recommended procedures for the detection of intestinal protozoa are (1) direct smears, (2) zinc flotation, and (3) permanent hematoxylin-stained slides.†

1. Direct Smears

Prepare at least 2 slides.

On one end of one slide, place a drop of saline.

On the other end, place a drop of D'Antoni's iodine (page 1821).

On the second slide, place 1 drop of D'Antoni's iodine at one end and at the other end a drop of 0.5% methylene blue in saline.

Emulsify small amounts of the stool in each drop.

Cover each drop carefully with a separate cover slip in such manner that the drops remain separated.

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*Warren F. Morrison, General Biological Supply House, Chicago, Ill. Personal communication.

†O. Felsenfeld, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, 1948, The C. V. Mosby Co.

Inspect the saline suspension immediately.

The other preparations may be examined after a few minutes (2 to 4), but before they dry.

If the examination is carried out quickly, a warm stage is not necessary.

2. Zinc Flotation

The technic of Faust and associates in the modification of Otto and co-workers* is used.

Mix a portion of the fecal specimen, about the size of a bean, with 33.1% zinc sulphate, specific gravity 1.18, in a small vial 5 cm. deep and 1.8 cm. in diameter.

Fill the vial with this solution until the meniscus is rounded over the edge of the tube.

Place a cover slip on the surface and let stand 1 hour.

Place a drop of D'Antoni's iodine on a slide and put the cover slip on it in such a way that the slide which lies on the surface of the stool-zinc sulphate emulsion rests on the drop of iodine.

Examine under a microscope.

3. Permanent Hematoxylin-Stained Slides

Any recommended procedure may be used. Slides fixed in Schaudinn's fluid are used for this staining. See pages 1823 ff. for the methods.

If the examination includes a search for helminths and their eggs, the formol-ether flotation is substituted for the zinc flotation.

The amount of material available for examination is decisive for the methods to be used. If only proctoscopic or rectoscopic swabs are available, permanent hematoxylin-stained slides are the sole method of investigation. Such specimens usually contain pus cells and macrophages which cannot be easily distinguished from protozoa in iodine-tinged or Quenzel-stained preparations. Formed stools more frequently contain cysts than trophozoites. Thus more emphasis must be laid on flotation technics when such specimens are studied. Formalin is a better preservative for cysts than PVA, which often causes shrinkage, but is excellent for keeping trophozoites unchanged.

The importance of permanent hematoxylin-stained slides cannot be emphasized enough. Frequently laboratory workers not too familiar with protozoology are compelled to make a decision if a patient does or does not harbor intestinal protozoa. If permanent preparations are available, one always can submit them to a parasitologist for consultation.

At least 5 consecutive stool specimens taken at least two days apart, collected during a time when no antibiotic or chemotherapeutic agents were given, must be examined before a patient is declared "negative" (Becerra). After treatment, weekly and later monthly examinations are recommended for one-half year.

FIXATIVES USED IN PROTOZOOLOGY

Schaudinn's Sublimate Alcohol Solution

95% alcohol	1 part
Concentrated watery solution of bichloride of mercury	2 parts

(With or without addition of a small amount of acetic acid)

This solution of bichloride of mercury is made by dissolving about 8 grams of the sublimate in 100 c.c. of boiling distilled water. After cooling, a few crystals separate out.

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*O. Felsenfeld and V. Young in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, 1948, The C. V. Mosby Co.

This is a very valuable solution. It is advisable to heat it to 50 to 60° C. before use. Smears remain in it 5 to 10 minutes, pieces of tissue according to size, one-half hour or longer.

Wash specimens in water; or, better, in 50% alcohol.

Carry over into iodine-alcohol, a 70% solution of alcohol to which tincture of iodine has been added until the color becomes light brown. Leave the smears in this solution for 15 minutes, then place for one-half hour in 70% alcohol. Wash and harden. Stain according to choice.

Carnoy's Solution

Absolute alcohol	6 parts
Chloroform	3 parts
Acetic acid	1 part

This is a good solution for preservation of cysts and spores, to fix protozoa in tissue. It is especially recommended to fix Arthropods. For embedding, the material can be put directly in chloroform and in concentrations of alcohol and paraffin. Stain according to choice.

Fleming's Solution

Chromic acid, 1%	15 parts
Osmic acid, 2%	4 parts
Acetic acid	1 part

For smears, time is 5 to 10 minutes; for small pieces of tissue, one hour to several hours.

Wash in water, then in 50 to 70% alcohol for hardening.

Stain with iron hematoxylin or aniline stains.

Herman's Platinum Chloride Osmic Acid Solution

Platinum chloride, 1%	15 parts
Osmic acid, 2%	4 parts
Acetic acid	1 part

This is a very good solution for differentiation of protoplasmic structures.

Picric Acetic Acid Solution

Concentrated watery picric acid solution	97 parts
Acetic acid	3 parts

To make the picric acid solution, add 1 gram picric acid to 100 c.c. of hot distilled water and filter after cooling.

This is designed for large protozoa, ciliates and Gregarina, one-half hour; and for large pieces of tissue, one to twelve hours.

After fixation, wash in 50 to 70% alcohol. For staining, use carmine and hematoxylin solution.

Bouin's Solution

Concentrated watery picric acid solution	15 parts
Formol (commercial 40% formaldehyde solution)	5 parts
Acetic acid	1 part

STAINING AMEBAE AND OTHER INTESTINAL PROTOZOA

Examination of Fresh Material

For the examination of fresh material it is necessary to use only a small amount of the fresh stool between the slide and cover glass. Examine with the low power and then with the oil immersion objective. In acute cases amebae are very numerous. The cysts are often mistaken for air bubbles by beginners. Differentiation must be made from Blastocystis. The addition of a solution of iodine one part, potassium iodide two parts, and water 100 parts, or 2% eosin solution, helps the examination. Eosin stains the background but does not stain the amebae and flagellates. Iodine stains the nuclei brown.

It is very important to examine for cysts. The Dutch investigators, Bayer and Kuenen, use a concentration method to demonstrate cysts. Another method by Riparte and Petit is as follows:

Copper chloride	1.0 gram
Copper acetate	1.0 gram
Formalin	50.0 c.c.
Camphorated water (1 part camphor, 2 parts alcohol, 200 parts water, filtered)	250.0 c.c.
Distilled water	700.0 c.c.
Acetic acid	6.5 c.c.

Rub up a hazelnut-sized piece of stool with 2 to 5 c.c. water, then add 50 c.c. of the above solution and shake. Filter through a gauze filter. Put it into a separatory funnel. Add 15 c.c. ether, shake, removing the stopper two or three times, and let stand. In a few seconds, the ether-impregnated detritus settles at the bottom. In this lower layer are found the cysts, and the eggs of worms, which can be concentrated by centrifugalization for a few minutes. The sediment can be examined fresh or stained.

LUGOL STAIN

Fresh Preparations Stained With Lugol Solution

Follow the same method as above, but first mix the drop of fecal suspension with a drop of strong Lugol solution, then cover with a cover glass.

Strong Lugol Solution

Metallic iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	50 c.c.

Dissolve the potassium iodide in as little of the water as possible, then add the iodine to this solution. Shake constantly while slowly adding the remainder of the distilled water. Filter through filter paper after 24 hours.

D'Antoni Iodine Stain for Wet Preparations of Intestinal Protozoa[1]

D'Antoni has given an excellent method of using potassium iodide for preparing an iodine stain for use in wet preparations containing intestinal protozoa.

To a 10 per cent standardized potassium iodide solution is added 1.5 gm. of powdered iodine crystals per 100 c.c., in order to provide an amount of iodine considerably in excess of saturation and to compensate for volatilization. The solution is allowed to stand for four days and is then filtered.

D'Antoni emphasizes the following points in the preparation of this stain:

1. Solution should be weighed to the fourth decimal place in standardizing the potassium iodide.
2. Iodine concentration will remain constant only if powdered iodine is used in the solution.
3. To insure saturation, the solution should be allowed to stand for four days before it is used.
4. The solution should always be filtered before being used.
5. Filtered bottles of solution should be kept in tightly closed glass-stoppered bottles for periods not exceeding four weeks. However, unfiltered stock solutions can be kept for long periods of time even if stoppered only lightly, and from these, filtered solutions can be made up at a moment's notice.

Wet preparations of the stool, either unconcentrated or concentrated, are stained as follows:

a. The Smear: To a large fecal slide add 2 drops of saline and, with a wooden applicator, smear on a small portion of feces until a consistency is reached where book print is still legible through the film. Place a cover slip over half of the smear and then place a drop of iodine stain on the uncovered half. Put a cover slip over this stained portion.

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[1] Am. J. Trop. Med. 17: No. 1, 1937.

b. The Concentrate: To the jar containing feces add a quantity of water equal to the amount of feces. Shake vigorously and filter through a single thickness of cheesecloth into a 13 by 100 mm. test tube. Centrifuge for 30 seconds, pour off the supernatant fluid, and add water until the tube is half full. Shake, and then pour off enough of it so that, on second centrifugation, a sediment will remain which covers the rounded portion of the bottom of the test tube. Add water and centrifuge the solution a second time. Discard the supernatant fluid, and add an amount of iodine which is about twice that of the sediment. Shake this mixture and pour directly on a glass slide, and, after placing a cover glass on top, examine the mixture.

This method stains *Endamoeba histolytica* very well, with the peripheral nuclear chromatin and central karyosome in very vivid contrast to the more darkly stained nonchromatin material. Glycogen masses are only slightly darker than the surrounding cytoplasm. Other protozoa stain equally well. Eggs are not overstained except those of *Ascaris lumbricoides* which are previously heavily bile stained.

D'Antoni found that powdered iodine was preferable to iodine crystals in making up his solution because, when powdered iodine was used, the saturation point was almost reached within four days and the solution remained constant even if allowed to stand in the open air. Neither of the above results was obtained if crystals were used.

It is important to prepare the 10% potassium iodide solution accurately. Because of its simplicity and accuracy, the specific gravity method is recommended for this standardization.

Preparation of Fecal Smears, Fixed, Wet, and Stained

Demonstration of Trophozoites of Intestinal Amebae— the Polyvinyl Alcohol (PVA) Technic

The PVA-Fixative Technic in the Diagnosis of Amebiasis.—

Donaldson[1] has given a summary of this method which furnishes the physician in the laboratory with a means of preserving these fragile organisms for a subsequent examination and study. He described a two-vial method of shipping specimens. In one vial is mixed a small portion of freshly passed stool with the PVA-fixative. The other vial is empty and should receive a small portion of the fecal specimen for other studies, such as fresh examination or cyst examination. Both vials should be sent to diagnostic laboratory with appropriate identifications.

Preparation of the PVA-Fixative.—

Reagents.—

1. Modified Schaudinn's Solution	
Glacial acetic acid	5.0 c.c.
Glycerol	1.5 c.c.
Schaudinn's solution (2 parts saturated aqueous solution of mercuric chloride and 1 part 95% ethyl alcohol)	93.5 c.c.
2. Polyvinyl Alcohol (PVA) Powder (Produced by E. I. du Pont de Nemours & Co., Inc., Electrochemical Department, Niagara Falls, N. Y., and designated as "Elvanol 90-25").	5 gm.

Heat the modified Schaudinn's solution to 75° C., and while mechanically stirring, add slowly the PVA powder. After cooling, the solution should be clear and free of lumps. The PVA-fixative remains satisfactory for several months and can be used either at room temperature or heated to 50° C.

Preservation of Specimens With PVA-Fixative.—

Specimens can be fixed and preserved either directly or on microscope slides or in vials. Fix the specimens immediately after passage from the patient, before the organisms lose their characteristic morphology.

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[1] J. Am. Med. Tech. 9: 70, July-Aug., 1949.

On microscope slides: A drop of the dysenteric stool or other material is placed on a microscope slide and mixed with three drops of the PVA-fixative. The mixture is then smeared over approximately two-thirds of the glass surface. The slide is maintained in a horizontal position until the film is thoroughly dried (preferably overnight at 37° C.). The dried films remain satisfactory for staining for many months.

In vials: A quantity of the specimen is introduced into a vial containing three or more parts of the PVA-fixative and thoroughly mixed. Smears for staining can be prepared immediately (or months later) by spreading a drop or two of the mixture over the surface of a slide. Do not have the smears too thick. Smears must be thoroughly dry.

Staining of the PVA Films.—

The long Heidenhain iron-hematoxylin procedure gives best results. The Tompkins-Miller[2] technic is a more rapid technic that gives uniformly good results and in most instances is satisfactory for diagnosis.

In all the hematoxylin procedures the dried films are first placed in the 70% alcohol iodine in order to remove the mercuric chloride crystals. Ten to 20 minutes in this solution is sufficient. The remaining steps depend upon what technic is being employed. Protect the stained film with a mounted cover-slip; however, the uncovered films can be satisfactorily examined with the oil immersion objective.

The advantage of this fixative technic is that it makes possible the successful staining of organisms occurring in fluid specimens. The solution serves as an adhesive as well as a preservative and prevents the loss of organisms during the staining procedure.

Heidenhain Iron-Hematoxylin Method

1. Fix in a mixture of 2 parts saturated aqueous solution mercuric chloride (corrosive sublimate) and 1 part alcohol, 20 to 60 minutes.[3] (Put specimen on cover glasses, not slides. Smear with needle. Float on surface of Petri dish containing fixative, face downward, then turn them around with forceps, face upward. They sink.)

2. Pour off sublimate alcohol; put back into bottle. Pour on idio-alcohol. (Alcohol 70% and tincture of iodine enough to give light port-wine color.)

3. Let stand 20 to 30 minutes. Pour off.

4. Place in 70% alcohol one hour or longer (24 to 48 hours). Pour off.

5. Iron alum 4%. This is *ferric* alum [$Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$]. One hour. Pour off.

6. Stain one hour in Heidenhain hematoxylin, 1%.

Heidenhain hematoxylin is made by dissolving 1 gram hematoxylin in 10 c.c. of 95% alcohol and diluting to 100 c.c. with distilled water. It should not be used for at least four weeks.

Wash in water. This should be thoroughly carried out using two separate containers holding at least one liter of water each. As James states, if the preparations are not thoroughly rinsed, an excess of the mordant is carried over into the hematoxylin solution, making decolorization difficult, and the amebae are likely to appear a dirty yellow with indistinct nuclei, instead of showing a clear gray cytoplasm with sharply defined and clear-cut black or deep purple nuclei.

James claims that it is not necessary, though advisable, to allow the hematoxylin solution to ripen for four weeks before using. If it is thoroughly aerated by allowing a current of air to pass through it overnight, it will be ready for use in the morning. The diluted solution should be filtered after use into a clean bottle. Later, this filtered diluted solution should be used with an equal part of the original stock solution, diluted one-half, then filtered again into the second bottle, and so on until the original stock solution is used, when there will be two bottles of stain. These can be used undiluted repeatedly. They improve with use until the hematoxylin is finally precipitated. This can be detected by adding a few drops of the stain to a test tube half full of tap water. If the resulting color is yellow or green, the stain is no longer useful; if the color is blue or violet, the stain is still good. Only a good quality of distilled water should be used for diluting the stock solution; tap water or alkaline water will precipitate the hematoxylin or, rather, the hematin, which is the active staining principle. The staining solutions should be kept in brown glass bottles, away from the light.

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[2] Am. J. Clin. Path. **17**: 755, 1947.

[3] Wm. M. James (J. A. M. A. **102**: 1175, 1934) has found that fixation in a modified Schaudinn's fluid, consisting of two parts of physiologic solution of sodium chloride saturated with corrosive mercuric chloride and one part of 96% or absolute ethyl alcohol, plus the addition of from 2.5 to 5% of glacial acetic acid, has given better results than the aqueous solution saturated with corrosive mercuric chloride, without the acetic acid. The weaker acetic acid solution is better for the delicate trophozoites in acute and subacute dysentery, and the stronger solution gives better fixation for cysts. He believes that from twenty to sixty minutes is too long a time for fixation of thin stool smears, since most authorities find that from five to ten minutes is ample. He prefers to fix at room temperature as the heated fixing solutions produce artefacts, as has been pointed out by Leland Cleveland.

7. Differentiate with 2% iron alum in a dish with a cross marked on the bottom to designate one, two, three, and four minutes' immersion. Take out the thinnest after one minute; the thicker specimens are differentiated longer.
8. Place in water.
9. Wash in running water for twenty minutes.
10. Place the various ingredients for the final dehydration in small bottles:

60% alcohol
80% alcohol
80% alcohol
96% alcohol
Absolute alcohol
Xylol
Xylol

11. Dehydrate as for paraffin sections.
12. Mount in Canada balsam or gum damar.

Ziegler Modification of the Heidenhain's Iron-Hematoxylin Method

This modification, described by Ziegler[1] and further emphasized by Tompkins and Miller,[2] embodies a basic change by the application of 2% aqueous phosphotungstic acid for differentiation. This gives uniform permanent film preparation which can be made more rapidly than by the preceding method.

Fixation.—

Prepare fresh fecal films on slides or cover glasses and fix while wet. Slides are more durable for routine use. Fixation while the film is wet and fresh is of considerable importance, because drying results in degeneration and distortion of the protozoa. The saline modification (see footnote on page 1823) by James is a very good departure.

Fix in Schaudinn fluid in saline solution, five minutes.

Remove residual mercury in 70% alcohol, with iodine added to obtain "port-wine" color, one minute.

Remove residual iodine in 70% alcohol, two changes, one-half minute each.

Mordant in 4% aqueous ferric ammonium sulphate, three to five minutes.

Wash in running tap water, one minute.

Stain in week-old 0.5% aqueous hematoxylin, one minute.

Remove excess stain by dipping in water.

Differentiate in 2% aqueous phosphotungstic acid, two minutes or more.

Place in tap water, one minute, until the hematoxylin is blue.

Dehydrate in 95% and in absolute alcohol, one minute each.

Clear in xylol, one minute.

Mount in gum damar.

Rapid Technic for Staining Amebae, Schaudinn

Use slides free of grease and oil. Make very thin smears. Do not allow the smears to dry at any stage of the process.

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[1] Arch. Path. 37: 68, 1944.
[2] Am. J. Clin. Path. 17: 755, 1947.

Reagents.—

Fixative.—

Mix 2 parts of warm Schaudinn fixative (saturated aqueous solution of mercuric chloride $HgCl_2$) and 1 part of absolute ethyl alcohol.

Iodine-Alcohol Mixture.—

Equal parts of iodine and 70% ethyl alcohol.

70% and 50% Alcohol.—

Iron Alum.—

Ferric ammonium sulphate	2 gm.
Distilled water	100 c.c.

Heidenhain Hematoxylin.—

Hematoxylin	1 gm.
Alcohol, 95% ethyl	10 c.c.

Dissolve and add 100 c.c. distilled water.

Xylol.—

Technic.—

Fix in the modified Schaudinn fixative (warm) for 2 minutes.

Place in iodine-alcohol for 2 minutes.

Place in 70% alcohol for 1 minute.

Place in 50% alcohol for 1 minute.

Wash in distilled water for 1 minute.

Place in *hot* iron alum for 2 minutes.

Wash in running tap water for 2 minutes.

Stain in Heidenhain hematoxylin (hot) for 1 minute.

Rinse in distilled water.

Decolorize in iron alum on a glass plate under the microscope until structure appears.

Wash in running tap water for 2 minutes.

Dehydrate in 50% alcohol for 1 minute, then in 70% alcohol for 1 minute, then 95% alcohol for 1 minute.

Complete the dehydration in 100% alcohol for 2 minutes.

Clear in xylol for 2 minutes.

Mount in gum damar or Canada balsam and examine with the oil immersion objective.

Staining Intestinal Protozoa, Modified Method of Cuendet[1]

Cuendet modified the original iron-hematoxylin technic, replacing the Heidenhain hematoxylin with a new staining solution.

Reagents.—

Stain.—

Alcohol, 95%	10 c.c.
Hematoxylin crystals (certified)	1 gm.
Glycerine, c.p.	10 c.c.
Distilled water	80 c.c.

Dissolve the hematoxylin crystals in the alcohol with a little heat, or by use of a mortar and pestle. Add the glycerine and the distilled water. If the solution is a deep red color it is neutral and no further treatment is necessary. If it is yellowish-brown to deep brown it is acid. Neutralize by adding 1% solution of potassium carbonate a drop at a time with constant shaking until the deep red color of the neutral solution is imparted to the foam. If the solution is purple to red-violet, it is alkaline. Neutralize by using N/5 hydrochloric acid. Expose the neutral staining solution in a clear glass bottle to daylight, allowing it to ripen for 2 or more weeks before using.

Schaudinn Fixative.—

Mix, freshly each day, 2 parts of saturated solution of mercuric chloride ($HgCl_2$) and 1 part of 95% alcohol.

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[1] Lab. Digest **10**: 8, 9-10, Jan., 1947.

Iodine-Alcohol Solution.—

70% alcohol with enough Lugol solution to give it a dark port-wine color.

Mordant.—

Dissolve 4 gm. ferric ammonium sulphate ($FeNH_4(SO_4)_2.12H_2O$ violet crystals) in distilled water and dilute to 100 c.c. with distilled water.

2% Ferric Ammonium Sulphate Solution (Iron Alum).—

Dissolve 2 gm. ferric ammonium sulphate in distilled water and dilute to 100 c.c. with distilled water.

Graded Alcohols, 50%, 70%, 80%, 95%, and 100%.—

Xylol.—

Gum Damar.—

Technic.—

The best consistency for feces from which smears are to be made is that of a thin porridge; smears are best made by using a soft bristle brush to spread the specimen. If concretions or solidly formed feces are presented for examination, proper consistency is obtained by grinding the material in a mortar with blood serum as a diluent. Smears are then made and placed immediately in hot (56° C.) Schaudinn's fixative.

Heat is an essential part of the technic. Inactivator temperature (56° C.) is ideal. Replace the water lost by evaporation of the solutions, from time to time, and occasionally replace the solutions.

Great care must be strictly observed so that smears are never allowed to dry at any time in the entire process, from the actual making of the smears to the final mounting.

Fix fresh wet smears in hot (56° C.) Schaudinn's solution for at least 2 minutes.

Remove smears, wipe the backs and edges dry with a clean cloth, dip in and out of iodine-alcohol solution until mercuric chloride is removed.

Wash the mercury-iodine compound from the smears in gently running tap water. This requires 1 or 2 minutes. To place slides in gently running tap water, hold the smear under a small stream of water from a faucet; or, better results are obtained and time is saved by introducing a small rubber tube between the slides in a Koplin jar and regulating the flow of water with the tap. A small battery of stop-cocks on a water line over a drainboard or sink will permit washing several jars of slides at once.

Place smear in hot (56° C.) mordant for 5 minutes. This step may require 2 to 5 minutes. The staining time should be the same as the time in the mordant. In most cases, 5 minutes' immersion in the mordant and 5 minutes in the stain give best results.

Remove smear from mordant and dip in distilled water. Place in hot (56° C.) staining solution for 5 minutes.

Remove and wash in gently running tap water as described above. Differentiate in 2% cold aqueous ferric ammonium sulphate (iron-alum). Dip the smear in and out of the iron-alum solution, and note when the excess stain has been removed. When this is accomplished, there will be only a trace of the stain visible in the drops of solution which fall from the slide. A little practice is necessary before this step is mastered.

After differentiation, wash in gently running tap water for 1 or 2 minutes. Dehydrate in graded alcohols, 50, 70, 80, 95, and 100%. Dry the back, edges, and other parts of the slide not containing the specimen each time the slide is transferred from one alcohol to another, then dip in and out of the alcohol until a thin, even film of alcohol covers the smear. If the smear looks streaked or oily, it still contains more water than alcohol, and more time is indicated for immersion in that alcohol.

Transfer the smear, after removing the excess alcohol, into xylol. Dip in and out until a thin, even film covers the spread surface. Transfer to a second jar of xylol and prepare the slide for mounting. Gum damar is preferred as a mounting medium because it will not turn yellow with age, and it dries and hardens quickly. Canada balsam may be used, however, although it hardens slowly, and preparations will become yellow within about four or five years.

Quensel Stain for Protozoa[1]

Reagent.—

Mix 20 c.c. of a saturated solution of Sudan III in 80% ethyl alcohol and 30 c.c. of filtered saturated methylene blue, medicinal solution.

Filter into 50 c.c. of 10% solution of cadmium chloride, c.p., in distilled water.

Shake gently for 20 minutes.

Filter.

Superimpose filter paper, residue up, on another filter paper, and leave overnight to dry.

Transfer precipitate to fresh filter paper and wash with 25 to 30 c.c. of distilled water.

Dissolve the washed precipitate in 250 c.c. distilled water.

If crystals form, filter.

Technic.—

Mix a small amount of the fecal material on a slide in a large drop of Quensel stain.

Cover with a cover glass.

Amebae stain various intensities of blue in 5 to 20 minutes. Nuclear walls and karyosomes show distinctly under high dry or oil immersion lenses.

According to Packard,[2] the stain is too concentrated. Intensity depends upon the methylene blue. It is good practice to make various dilutions of the methylene blue in water and determine the final dilution in that manner. Diluting to 500 c.c. instead of to 250 c.c. is suggested by Packard.

A Rapid Method for Preparing Permanent Slides of Intestinal Parasites

Method of Johnson[3]

Reagents.—

95% Ethyl Alcohol.—

Schaudinn's Fixative.—

Prepare a saturated aqueous solution of mercuric chloride, c.p., and filter through filter paper.

To each 100 c.c. of this solution add 50 c.c. of 95% alcohol.

Before using, add 20 c.c. of glacial acetic acid, c.p., to each 150 c.c. of the mixture. Use the fixative only once.

4% Iron Alum.—

See p. 1823. This solution can be used for several weeks provided it is not cloudy and does not contain a precipitate.

0.25% Iron Alum.—

Prepare fresh from the 4% solution by pipetting 12.5 c.c. of the 4% solution into distilled water and diluting to 200 c.c. with distilled water. This must be used within 24 hours after it has been diluted.

0.5% Hematoxylin.—

Dissolve 1 gm. hematoxylin (certified)
in 10 c.c. of absolute alcohol.

Dilute to 200 c.c. with distilled water.

Allow to stand for 6 weeks in an Erlenmeyer flask loosely plugged with cotton. The solution must be exposed to sunlight.

Technic.—

Under no conditions must the slides be allowed to dry during the procedure.

Place all solutions in Koplin jars.

Prepare thin smears from fresh, warm (recently voided) stools or rectal swabs, on clean slides. Always prepare several smears from each specimen.

Immerse in Schaudinn's fluid. Keep the jar on a water bath during the fixation at 60° C., for 10 minutes.

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[1] Manual of Tropical Medicine, Philadelphia, 1945, W. B. Saunders Co.

[2] Fridel Packard, New York City: Personal communication.

[3] Am. J. Trop. Med. 15: 551, 1935.

Transfer to 95% alcohol containing enough tincture of iodine to give it a rich port-wine color—5 minutes.

Transfer to 70% alcohol—5 minutes.

Rinse with tap water—3 minutes.

Immerse in 4% iron alum—15 minutes.

Rinse with tap water—2 minutes.

Stain with 0.5% hematoxylin—10 minutes.

Immerse in 0.25% iron alum—12 minutes.

Dehydrate by passing the slides through 50%, 75%, 85%, 95%, and absolute alcohol 5 minutes in each jar.

Clear in xylol—5 minutes.

Mount in Clarite under a cover glass.

Method of Ratcliffe, Modified by Felsenfeld and Young*

Reagents.—

Schaudinn's Fixative (above).

Mallory's Phosphotungstic Acid Hematoxylin.—

Phosphotungstic acid, 10% aqueous	10.0 c.c.
Haem-ammonium	0.1 gm.
Distilled water	90.0 c.c.

Age one to three weeks before use.

Technic.—

Make smears on slides and place them in a Koplin jar containing Schaudinn's fluid. Leave them in the fixative for at least 4 hours, but not longer than 48 hours.

Carry out the staining in Koplin jars.

Transfer to 95% alcohol containing enough tincture of iodine to give it a rich port-wine color—5 minutes.

Transfer to 75% alcohol—5 minutes. The slides may remain overnight in this alcohol, if necessary.

Distilled water—5 minutes.

Mallory's hematoxylin, at least 30 minutes. The slides may remain in this stain overnight or for as long as 48 hours.

Distilled water until the smears become blue. This usually requires 5 to 12 minutes.

Dehydrate in 70%, 95%, and absolute alcohol, 5 minutes each.

Clear in xylol—5 minutes.

Mount under a cover glass with Clarite.

Gram Stain

The Gram stain is highly recommended by Kourí for staining amebae.

Rapid Permanent-Mount Stain for Intestinal Protozoa

Method of Lawless†

Reagent.—

Acetone	50 c.c.
Glacial acetic acid	50 c.c.
Formaldehyde solution USP	10 c.c.
Schaudinn's solution (page 1819)	890 c.c.

Add to this solution 1.25 gm. of acid fuchsin
and 0.5 gm. fast green FCF.

Mix.

The solution is ready for use.

Store in a tightly stoppered bottle, preferably a brown one.

The solution remains stable for at least two months or more.

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*Res. Clin.-cient 14: 11, 1945.

†Am. J. Trop. Med. & Hyg. 2: 1137, 1953.

Technic.—

Using an applicator stick, spread a thin layer of fecal material on a glass slide. At no time during the staining procedure must the fecal smear be allowed to dry.

Completely cover the preparation with the reagent, using a medicine dropper or a dropping bottle with a glass stopper.

Heat gently over a burner until steam is first observed. Two or three slow passes of the fecal smear through a flame is usually sufficient. Do not boil and do not allow staining solution to flame.

Wash immediately, gently, under tap water.
Place in 50% alcohol for 30 seconds.
Place in 70% alcohol for 30 seconds.
Place in 95% alcohol for 15 seconds.
Place in absolute alcohol for 15 seconds.
Clear in xylol for 1 minute.
Mount in gum damar or Canada balsam.

Staining Reaction.—

The staining characteristics of the intestinal protozoa are similar to those with iron hematoxylin. Nuclear detail and chromatoid substance are particularly clear. Cysts of all species stain well, and also the trophozoites.

The background should be bluish-purple, the parasites a variable blue to purple.

CULTURE METHODS FOR ISOLATION OF PATHOGENIC AMEBAE

A number of different culture methods have been suggested for the isolation of the pathogenic amebae. Bayless, of Cleveland, has compiled the following condensed review of the facts on the various methods for cultivation:

1. Liver infusion agar covered with horse-serum-saline diluted 1:6.
2. Coagulated egg slant covered with horse-serum-saline or egg-white-saline.
3. Dehydrated blood serum with egg-white-saline or horse-serum-saline.
4. Coagulated egg slant covered with defibrinated blood in saline.
5. Inactivated human serum diluted with Locke's solution.
6. Liver infusion with hydrolyzed hemoglobin or washed red blood corpuscles.
7. Loeffler's serum slant covered with horse-serum-saline.
8. Three per cent hemoglobin agar covered with horse-serum-saline.

Embryo juice, ascitic fluid, testicle broth may be used instead of serum.

Observe sterile precautions.

Inoculation: with exudate, stool concentrate, washed cysts or sediment from culture.

Incubation: optimum 35° C. but a fall of a few degrees is safer than a rise above this point.

Subculturing interval: varies with strain and medium usually after 48 to 96 hours.

Survival: varies with different media; cysts washed and stored in saline survive indefinitely at ice box temperature.

Examination: the use of the warm stage gives best results.

Boeck and Drbohlav Medium No. 1

Cultivation of *Endamoeba histolytica* was first accomplished by Boeck and Drbohlav.[1] This culture medium consists of

Agar	14 grams
Salt	6 grams
Distilled water at 45° C.	900 c.c.

Mix one part with two parts of blood that has been heated for one-half hour at 100° C. with the addition of 1 per cent grape sugar.

Boeck and Drbohlav Medium No. 2

Locke's solution.—

Distilled water	1000.0 c.c.
Sodium chloride	9.0 gm.
Calcium chloride	0.2 gm.
Potassium chloride	0.4 gm.
Sodium bicarbonate	0.2 gm.
Glucose	2.5 gm.

Sterilize in the autoclave at 15 pounds pressure for thirty minutes.

Locke's Serum-Egg Medium.—

Mix the whites and yolks of four eggs thoroughly. Add 50 c.c. Locke's solution.

Mix thoroughly and filter through gauze.

Place in 5 c.c. quantities in 15 c.c. homeopathic vials. Pound in number 4 corks. Screw on the caps. Slant in the autoclave and sterilize at 15 to 20 pounds pressure for thirty minutes.

Slants should have surfaces 1 or 1½ inches long.

Preparation of the Blood Serum.—

Inactivate the serum by heating in a water-bath at 56° C. for one-half hour.

If the serum is not sterile, dilute with 2 or 3 parts of Locke's solution.

Filter through a Berkefeld filter, and test the filtrate for sterility.

Serum prepared in this manner may be placed in sterile ampules and sealed hermetically. These ampules may be kept in the refrigerator indefinitely.

Preparation of the Medium for Inoculation.—

Dilute the blood serum so that the final concentration of serum and Locke's solution is 1 part serum to 8 parts Locke's solution.

Cover the tubes with this mixture to a depth of 1 cm. above the slant.

Incubate for sterility before using.

Medium of Cleveland and Sanders[2]

Dissolve 33 gm. of Difco Endamoeba medium in 1000 c.c. of distilled water.

Distribute into tubes in quantities sufficient to make a medium slant with no butt.

Sterilize in the autoclave at 15 pounds pressure for 20 minutes.

Allow to harden in the desired position.

Add a mixture of 1 part horse serum and 10 parts of phosphate buffer pH 8.0 to each tube to a height of three-fourths of the slant.

Before inoculation, add 1 or 2 loopfuls of sterile rice powder.

Medium of Balamuth[3]

To 36 gm. dried egg yolks add an equal volume of distilled water.

Mix well.

Add 125 c.c. of 0.8% sodium chloride solution. Mix thoroughly.

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[1] Am. J. Hyg. 5: 371, 1925.

[2] Am. J. Hyg. 23: 114, 1936.

[3] Am. J. Clin Path. 16: 380, 1946.

Boil for 10 minutes, stirring constantly.

Dilute to the original volume with distilled water.

Press through a double layer of muslin.

Dilute to 125 c.c. with 0.8% saline solution.

Sterilize in the autoclave at 15 pounds pressure for 20 minutes.

Cool to a temperature below 10° C. and filter through a Buchner funnel using medium grade filter paper.

Add an equal amount of phosphate buffer pH 7.5 to the yolk mixture.

Phosphate Buffer, pH 7.5.—

Mix 43 c.c. of 17.48% dipotassium phosphate solution
and 7 c.c. of 136.09% monopotassium phosphate solution.
Add 14 parts of distilled water to 1 part of this mixture.

Prepare a solution of Wilson or Lilly liver concentrate by making a 5% suspension, filtering, and autoclaving.

Add 1 part of the liver extract to 9 parts of the buffered yolk infusion.

Distribute in tubes in 7 to 10 c.c. quantities.

Sterilize in the autoclave for 20 minutes at 15 pounds pressure.

Before use add a loopful of sterile rice powder.

The medium can be prepared from fresh egg yolks as follows:

Boil 4 eggs for 15 minutes.
Discard the whites.
Add to the yolks 125 c.c. of 0.8% saline solution.
Proceed as above, except that a routine filter paper may be used for filtration through the Buchner funnel.

COMPLEMENT FIXATION TEST FOR DIAGNOSIS OF AMEBIASIS

Method of Bozicevich, Hoyem, and Walston[1]

Because of the complex nature of the antigens used in complement fixation tests for helminthic and protozoan disease, the tests are often unreliable, a result due mainly to the anticomplementary effect of the antigen. The method described here eliminates most of the difficulties previously encountered. This test is similar in nature to the technics of Wadsworth,[2] Wadsworth, Maltaner, and Maltaner,[3] and Kent, Bukantz, and Rein.[4] The complement titer is determined on the basis of that amount which will give 50 per cent hemolysis when compared to the color standard. The test is conducted in a total volume of 1 c.c. in which 4 or these 50 per cent units are used.

Reagents.—

2 Per Cent Cell Suspension.—

In order to determine the end point for 50 per cent hemolysis, it is necessary, before the optimal hemolysin titer is determined, to prepare a 2 per cent suspension of cells from which a color standard is made.

Collect sheep cells by using sodium citrate, or by defibrinization. If the cells are to be held over 3 days, remove a portion from the original collecting container and wash 4 times with physiologic saline. If it is necessary to perform titrations with the same cell suspension for a period of several weeks, it is advisable to use Alsever and Ainslie's[5] solution as recommended by Bukantz, Rein, and Kent[6] for the preservation of cells to reduce fragility and consequent hemolysis. After the final washing, fill a 15 c.c. graduated conical centrifuge tube with washed cells and centrifuge at 2,000 r.p.m. for 10 minutes. Measure the quantity of packed cells and multiply the volume occupied by the cells by a factor of 50 to make a 2 per cent suspension. This gives the total volume which must be made up with saline. Thus, if the volume of packed cells is 1.5 c.c., 1.5 × 50 = 75 c.c. Pour the contents of the tube into a graduated cylinder and wash the centrifuge tube with saline, adding the washings to the graduated cylinder. Add saline until a volume of 75 c.c. is reached.

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[1] Pub. Health Rep. 61: 15, April 12, 1946; Lab. Digest 10: Sept., 1946.

[2] Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Baltimore, 1927, Williams and Wilkins Co., 704 pp.

[3] J. Immunol. 35: 217, 1938.

[4] J. Immunol. 53: 37, 1946.

[5] New York J. Med. 41: 126, 1941.

[6] J. Lab. & Clin. Med. 31: 394, 1946.

Hemoglobin Standard.—

Pipette 10 c.c. of the 2 per cent cell suspension into a graduated centrifuge tube and pack in the centrifuge at 2000 r.p.m. for 10 minutes. Without disturbing the cells withdraw most of the supernatant fluid by means of a capillary pipette. Add distilled water up to the mark 9.5, lake the cells completely by shaking, and add 0.5 c.c. of a stock buffer solution or 0.5 c.c. of a 17 per cent saline solution to restore tonicity.

Stock Buffer Solution.—

Sodium chloride (NaCl)	17.00 gm.
Sodium phosphate, dibasic (Na_2HPO_4)	1.13 gm.
Potassium phosphate, monobasic (KH_2PO_4)	0.27 gm.
Distilled water to make	100 c.c.

This stock solution may be used in place of saline as a diluent for all the reagents by adding 1 part of the stock solution to 20 parts of distilled water.

Color Standard.—

Prepare the color standard from the standard hemoglobin solution in tubes of the same diameter as those which are to be used in the test proper.

Note.—Since the total volume in the test is 1 c.c., all the titrations are conducted on the same volume and the measurements of the reagents given in all the tables are in tenths of a cubic centimeter.

TABLE 173.—PREPARATION OF COLOR STANDARD

	PER CENT OF HEMOLYSIS										
	0	10	20	30	40	50	60	70	80	90	100
Saline	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Standard hemoglobin	0.00	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18	0.20
2 per cent red cells	0.20	0.18	0.16	0.14	0.12	0.10	0.08	0.06	0.04	0.02	0.00

Shake the tubes and label according to the degree of hemolysis. Then centrifuge at 2,000 r.p.m. for 10 minutes.

Titration of Hemolysin.—

It is advisable to begin the titration of hemolysin with a 1:2000 dilution for, if the pooled hemolysin does not attain this titer, it is not satisfactory and the animals should be given further immunization. To prepare the sensitized cells for this titration, make up the hemolysin dilution desired in 10 c.c. amounts and add an equal volume of the 2 per cent cell suspension. The "sensitized cells" must be prepared 15 minutes before using them in the test. Prepare a 1:100 dilution of complement from the pooled sera of several guinea pigs. Arrange the tubes from the initial titration of hemolysin according to Table 174. In this preliminary titration of hemolysin, the complement titration is performed at the same time.

TABLE 174.—HEMOLYSIN TITRATION

	TUBE NUMBERS									
	1	2	3	4	5	6	7	8	9	10
Saline	0.55	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10
Complement	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50
Sensitized Cells	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40

Make this arrangement for each dilution of hemolysin to be tested. Place the rack in a 37° C. water bath for 30 minutes, remove, and centrifuge the tubes at 2,000 r.p.m. for 10 minutes. Compare each tube with the color standard tubes and record the results in per cent of hemolysis according to the plan given in Table 175.

The optimal dilution of hemolysin as defined by Wadsworth[2] is that dilution beyond which further increase in the concentration of hemolysin does not appreciably change the quantity of complement required for 50 per cent hemolysis. Therefore, from this titration it is apparent that the optimal dilution of hemolysin is 1:3000. This concentration is used henceforth for the daily titration of complement and for the sensitization of red cells for the test proper. This titration serves a dual purpose in this instance, in that both the complement and hemolysin titration are conducted at the same time.

Titration of Complement.—Titrate the complement daily with the optimal dilution of amboceptor as indicated in Table 175. Dilute fresh pooled sera, obtained from several guinea pigs, 1:100 and titrate according to Table 174. In this titration use only the optimal dilution of hemolysin. After incubation and centrifugation, compare each tube in the test to the 50 per cent hemolysis tube in the color standard. The color standard must be prepared fresh daily with a portion of the cells from the same lot which is to be used in the test proper. If the color standard tube containing the 50 per cent hemolysis matches exactly Tube 3 of the titrations containing 0.15 c.c. of 1:100 complement, Tube 3 is regarded as 1 unit of complement. If the 50 per cent color standard falls between 2 tubes, it is necessary to interpolate the 1 unit of complement. Since 4 units of complement are required in each 0.20 c.c., make the dilution of complement according to the following formula:

TABLE 175.—READING OF THE HEMOLYSIN TITRATION

HEMOLYSIN DILUTIONS	TUBES									
	1	2	3	4	5	6	7	8	9	10
1:7000	0	0	0	0	5	10	20	25	35	45
1:6500	0	0	0	8	20	30	40	55	65	68
1:6000	0	0	3	10	25	35	50	60	65	70
1:5500	0	0	5	15	35	45	55	70	80	85
1:5000	0	0	8	30	50	60	80	85	90	95
1:4500	0	0	20	40	60	70	85	88	95	98
1:4000	0	0	25	50	75	80	85	90	95	100
1:3500	0	10	30	60	80	85	95	100	100	100
1:3000	0	30	55	80	90	95	98	100	100	100
1:2500	0	35	60	85	93	98	100	100	100	100
1:2000	2	37	65	90	95	100	100	100	100	100

4 × 0.15 = 0.60 c.c. of 1:100 complement. Consequently $\frac{0.60}{100} = \frac{0.20}{X}$. $X = 33.3$. Therefore, 0.20 c.c. of a 1:33.3 dilution of complement will be required for the test. This dilution is also used in the controls in order to determine quantitatively the amount of complement which may deteriorate in the presence of serum, antigen, and saline alone.

Technic of Diagnostic Test.—

Inactivate the serum for 30 minutes at 56° C. just prior to use. If the serum was inactivated the day before, it is advisable to reheat it at this temperature for about 5 minutes before using it. Use two dilutions of serum in the test, 1:5 and 1:10. Arrange the tubes and add the reagents in the order given in Table 176.

TABLE 176.—THE TEST PROPER

REAGENTS	TUBE NUMBERS										
	1	2	3	4	5	6	7	8	9	10	11
Saline*	---	---	0.30	0.25	0.20	0.30	0.25	0.20	0.30	0.25	0.20
Serum 1:5	0.20	---	0.20	0.20	0.20	---	---	---	---	---	---
Serum 1:10	---	0.20	---	---	---	0.20	0.20	0.20	---	---	---
Complement 4 units	0.20	0.20	0.10	0.15	0.20	0.10	0.15	0.20	0.10	0.15	0.20
Antigen	0.20	0.20	---	---	---	---	---	---	0.20	0.20	0.20

*Or diluted buffer.

Place the tubes in the refrigerator overnight (15 to 18 hours) at 5° C. The next day, add to each tube 0.40 c.c. of sheep cells which have been sensitized 15 minutes previously. Incubate in a 37° C. water bath for 30 minutes. Remove and read.

Endamoeba histolytica antigen is prepared by a modification of the technic of Rees, Bozicevich, Reardon, and Jones.[1] It is used for the complement fixation test for amebiasis and has proved to be useful in the differential diagnosis of liver abscesses and questionable cases of liver involvement.

Interpretation of Results.—

In the usual complement fixation technic, according to Bozicevich and associates,[1] the amount of complement which will deteriorate overnight in the presence of serum or antigen is not taken into consideration. With the method described here, it can be seen that the amount of deterioration which might occur with any given serum sample is taken into consideration in evaluating the final results of the test. For the purpose of convenience this deteriorating factor is called the binding power of the serum or of the antigen. In Table 176, Tubes 3, 4, and 5 contain, respectively, 2, 3, and 4 50-per cent units of complement to determine the binding power of the 1:5 serum dilution. Tubes 6, 7, and 8 containing, respectively, 2, 3, and 4 50-per cent units of complement should give the amount of complement bound in the 1:10 serum dilution. Tubes 9, 10, and 11 should show the quantity of complement bound by the antigen used in the test. Thus, it can be seen that the binding power is determined for both dilutions of serum and the antigen used in the test if any binding should exist. It is necessary to conduct binding powers on every single serum used in the test, but it is necessary to conduct only one set of binding powers for the antigen for the particular day that the antigen is used.

Remove the tubes containing 30, 50, 70, and 100 per cent cells, respectively, of the color standard and shake thoroughly. Regard any tube in the test matching the tube containing 30 per cent cells or more as a positive, and any tube showing less than 30 per cent fixation as negative.

Bozicevich and associates[2] give a few examples to demonstrate the advantage of the present method of conducting the complement fixation test. If a serum should give the following fixation results, it is apparent from Tubes 3, 4, and 5 that the serum itself will bind 3 units of complement. Yet with the usual methods, Tube 5 would be the control tube and the test would have been regarded as satisfactory. However, the second serum dilution 1:10 also has some binding power as disclosed by Tube 7. This serum is regarded as a negative when the present method of complement fixation is used. If the serum showing the binding displayed in the above example has any antibodies present the titer would be much higher.

TABLE 177

	TUBE NUMBERS										
	1	2	3	4	5	6	7	8	9	10	11
1:5 serum	++++		++++	+++	–						
1:10 serum		+				++++	+	–			
Antigen									–	–	–

At times, it has been found that antigen will begin to deteriorate and tests are made to guard against this feature as shown by Table 178.

TABLE 178

	TUBE NUMBERS										
	1	2	3	4	5	6	7	8	9	10	11
1:5 serum	++++		–	–	–						
1:10 serum		++++				–	–	–			
Antigen									++++	+++	–

In this case, it is apparent that the antigen, as disclosed by Tubes 9 and 10, binds at least 3 units of the complement by itself. Again, Tube 11, which is the usual control in other tests, is negative and the antigen would have been regarded as satisfactory. In this instance, it is necessary to repeat the test with a new and satisfactory antigen.

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[1] Am. J. Trop. Med. 22: 581, 1942.

[2] See footnote, page 1831.

In the example, Table 179, the test is regarded as positive.

TABLE 179

	TUBE NUMBERS										
	1	2	3	4	5	6	7	8	9	10	11
1:5 serum	++++		++++	++	±						
1:10 serum		++++				+++	±	-			
Antigen									-	-	-

The test is regarded as positive because the serum gave good fixation in the 1:10 dilution and in this dilution the anticomplementary effect of the serum is practically removed. The 1:5 dilution could not be read safely because of the strong anticomplementary tendencies.

Another Complement Fixation Test for Amebiasis[1]

A micro-Kolmer technic as modified by Microbiology Reference Laboratory, Veterans Administration Hospital, Bronx, N. Y., is here described.

Reagents.—All glassware must be scrupulously clean.

Sheep Cell Suspension.—

Collect sheep cells in sodium citrate or by defibrination.

Wash four times in physiologic saline solution (see page 2048). If the cells are to be used for several weeks, use preserving solution.

Fill a 15 c.c. graduated conical centrifuge tube with washed cells and centrifuge at 2,000 r.p.m. for 10 minutes.

Measure the volume of packed cells after removing the supernatant fluid.

Multiply by 50 to obtain the total volume of diluent required to make a 2% suspension. Suspend in Kolmer saline.

Kolmer Saline.—

To every 1,000 c.c. of 0.85% sodium chloride solution
add 100 mg. of magnesium sulphate.

Serum.—

The serum must not be chylous. Preferably withdraw from a fasting patient. If the serum is to be stored in a freezer and thawed shortly before the test is run, aseptic technic is not essential. Repeated thawing and freezing will render the serum anticomplementary if it has not been aseptically collected.

After the blood has clotted, loosen the clot, centrifuge, and remove the supernatant serum.

Inactivate at 56° C. for 30 minutes to destroy the natural complement.

Antigen.—

Obtain *Endamoeba histolytica* antigen. At present this is in the experimental stage and is not available.

Dilute with Kolmer saline 1:5. Use only freshly prepared dilutions.

Complement.[2]—

Bleed guinea pigs from the heart. Do not use pregnant pigs.

Allow the blood to flow gently from the syringe into a saline-rinsed bottle. Avoid rupturing the cells.

Refrigerate for 4 to 6 hours.

Decant supernatant fluid and centrifuge to remove blood cells.

Decant the serum into small rubber-stoppered test tubes, each containing exactly 1 c.c.

Freeze and store in a frozen state for at least one day before titrating.

Carry out the titration as indicated below (page 1836).

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[1] This test is still in the experimental stage. It is presented by permission of Michael Kenney, M.D.

[2] Prepared as for the Bengston technic, Pub. Health Rep. 59: 402, 1944, except that it is diluted 1:30 for hemolysin titration and 1:40 for complement titration.

For the hemolysin titration, dilute 1:30. Dilute 1:40 for the complement titration.

Perform the complement titration with the rack of tubes, the complement, antigen, and Kolmer saline all standing in an ice-water bath.

Deliver the Kolmer saline and the 1:40 complement with 0.2 c.c. pipettes, but never measure the volumes with the tip of the pipette.

Deliver the antigen and the amboceptor-red cell mixture with 1 c.c. pipettes.

To dilute the complement (the same stock as used in the hemolysin titration) 1:40, add 0.2 c.c. to 7.8 c.c. of cold Kolmer saline.

Sensitize the 2% sheep cells with the titrated hemolysin by mixing equal proportions of each, 15 minutes before needed, and placing in a refrigerator until used in the test.

Set up the complement titration in 10 by 75 mm. tubes.

Hemolysin.[3]—

Give rabbits 5 or 6 intravenous injections, at about 3-day intervals, of washed, packed sheep cells suspended in 10 c.c. of saline.

The initial dose is 0.5 c.c. of cells, followed by three doses of 1.0 c.c., and finally 1.5 c.c.

Bleed 7 to 9 days after the last injection of a preliminary titration gives a titer of 0.15 c.c. or more of the 1:250 dilution of hemolysin. (See page 2040 for method of bleeding a rabbit.)

Allow the blood to clot, and centrifuge.

Remove the supernatant serum (it need not be inactivated), and preserve with an equal part of best grade, neutral glycerin.

Store in a refrigerator.

For the titration, complement must be diluted 1:30 instead of 1:40.

Titration of Hemolysin

Prepare a 1:250 dilution of hemolysin by diluting 0.2 c.c. of the glycerinated serum to 50 c.c. with saline.

TUBE NUMBER	1	2	3	4	5	6	7	8	9	10
Hemolysin	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
Saline	0.55	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1
2% Sheep cells	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Shake and allow to stand 10 minutes at room temperature.

Add 0.2 c.c. (1⅓ units) of complement to each tube; shake.

Incubate in a water bath at 37° C. for 1 hour.

Find the smallest quantity of hemolysin which produces complete hemolysis.

Divide this by the dilution 250, which gives the amount of hemolysin providing *one unit.* Two units, or twice this amount, are used in the test.

Dilute the hemolysin so that 2 units are contained in 0.2 c.c.

The amount of hemolysin providing 2 units × the number of doses needed for the test = the c.c. of hemolysin needed. The number of doses × 0.2 c.c. = the total volume of the dilution.

Titration of Complement

Dilute the complement 1:40.

TABLE 180

TUBE	1	2	3	4	5	6	7	8	9	10
Complement (c.c.)	0.10	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.00
Kolmer saline (c.c.)	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13	0.15
Antigen (c.c.)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Shake well and place in a water bath at 37° C. for 1 hour.										
Sensitized sheep cells (c.c.)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Shake well and place in a water bath at 37° C. for 30 minutes.										

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[3] Bengston technic except for the dilution of complement.

Read. Find the smallest amount of complement which gives complete hemolysis. If all the tubes other than the control show hemolysis, the complement is hyperactive and should not be used.

For the test, dilute the complement so that 2 exact units will be represented in 0.1 c.c., according to Table 181.

TABLE 181

TUBE NO.	COMPLEMENT 1 EXACT UNIT	COMPLEMENT 2 EXACT UNITS	DILUTION RATIO
1	0.10	0.20	1:20
2	0.09	0.18	1:22.2
3	0.08	0.16	1:25
4	0.07	0.14	1:28.6
5	0.06	0.12	1:33.3
6	0.05	0.10	1:40
7	0.04	0.08	1:50
8	0.03	0.06	1:66.6
9	0.02	0.04	1:100
10	Control	Control	Control

Note: The dilution (D) is calculated as follows:

$$D = \frac{40 \times 0.1}{2\ U} \text{ when } U = \text{the exact unit of complement.}$$

Example: If tube No. 7 is the exact unit, then

$$D = \frac{40 \times 0.1}{0.08} = \frac{4}{0.08} = 1{:}50 \text{ dilution.}$$

Technic of the Test

(A) **Preliminary Serum Dilutions.—**

Make serial twofold dilutions in 10 by 75 mm. tubes.

TUBE	1	2	3	4
Kolmer saline	0.2	0.2	0.2	0.2
Inactivated undiluted patient's serum	0.2	0.2 from tube 1	0.2 from tube 2	0.2 from tube 3
Dilution	1:2	1:4	1:8	1:16

Use a 1 c.c. pipette to measure the saline, and a 0.2 c.c. pipette for the serial transfers.

(B) **Final Quantitative Test.—**

All racks must be set in an ice bath while performing the test.

TABLE 182

TUBE	1	2	3	4	5	6	7	8	9	10
Patient's serum (c.c.)	0.05 undiluted	0.05 undiluted	0.05 of 1:2 dilution	0.05 of 1:4	0.05 of 1:8	0.05 of 1:16	---	---	---	---
Kolmer saline (c.c.)	0.10	---	---	---	---	---	0.05	0.15	0.15	0.25
Titrated complement (c.c.)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	---	---
Antigen (c.c.)	---	0.10	0.10	0.10	0.10	0.10	0.10	---	0.10	0.10
Shake well and place in a refrigerator overnight.										
Sensitized sheep cells (c.c.)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Shake well and place in a water bath at 37° C. for 30 minutes. Take first reading after 15 minutes.										

Tube 1 is the patient's serum control. It must show complete hemolysis.

Tube 7 is the antigen control and must show complete hemolysis.

Tube 8 is the hemolytic system control and must show complete hemolysis.

Tube 9 is the control to test for the hemolytic property in antigen and must NOT show hemolysis.

Tube 10 is the cell control and must NOT show hemolysis.

Controls 7 to 10 must be set up with every batch of tests.

Compare the readings with a **color scale** prepared by using different proportions of sheep cell dilution and hemolyzed sheep cells:

1 part of 2% sheep cells + 6 parts of Kolmer saline for the sheep cell dilution, and sparkling tubes from hemolysin titration for the hemolyzed sheep cells.
Color scale:

TUBE	1	2	3	4	5
Sheep cell dilution	0.4	0.2	0.1	0.04	---
Hemolyzed sheep cells	---	0.2	0.3	0.36	0.4
Reading	4 plus	3 plus	2 plus	1 plus	Negative

The complement fixation test for amebiasis, as presented here, was used by Kessel[1] with the following results in the first 100 cases thus tested. Positive stool and positive complement fixation tests were found in 48 patients, or 48 per cent. Negative stool and negative complement fixation test were found in 32 patients, or 32 per cent. Negative stool and positive complement fixation test were found in only 6 patients, or 6 per cent, and positive stool with negative complement fixation test in 14, or 14 per cent.

Of the 10 control cases, 8 or 80 per cent had negative stools and negative complement fixation tests; 2 or 20 per cent had negative stools and positive complement fixation tests.

These data demonstrate that in 80 per cent there is a correlation of stool findings and complement fixation results, while false positive complement fixation tests were present in only 6 per cent, and positive stools with negative complement fixation tests in 14 per cent.

To the best of our knowledge, this is the first time that a similar antigen has been prepared and used successfully with the 100 per cent hemolysis system (Bengston technic). The technic used by Rees and his associates has involved the use of the 50 per cent hemolysis system which is considerably more complicated than the Bengston technic.

Although only 100 cases have been completely studied thus far, Kessel has found 80 per cent positive correlation with stool examinations with only 6 per cent false positives. A false positive has been defined arbitrarily as one with a positive complement fixation test and three negative stools. Since it is common experience for amebae to be discovered only after 5 or more stools have been examined, as in the cases discussed, further stool examinations might have reduced even more the percentage of false positives. Most encouraging were those patients with ulcerative colitis who had negative stools and negative complement fixation tests with no false positives. In view of the relative high specificity, minimal false positive reactions, the simplicity of the technic, and the availability of the antigen, this technic is considered a valuable adjunct to the armamentarium in the diagnosis of amebiasis.

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[1] Personal communication. For full details see Kessel, John J., Parrish, Margaret, and Parrish, Glenn: Am. J. Trop. Med. & Hyg. 3: 440, 1954.

MEDICAL PROTOZOOLOGY

Protozoa are unicellular animal organisms which are capable of carrying on in one cell all the biological phenomena necessary for life. **Metazoa,** on the other hand, are multicellular animal organisms which contain cells specialized for definite functions.

Protozoa are composed fundamentally of a protoplasmic mass containing one or more nuclei. The **cytoplasm** is fine or coarsely granular, and is composed of an external hyaline portion called **ectoplasm** and an internal granular portion or **endoplasm.** Distinct prolongations of the ectoplasm are closely related to the functions of movement and motility.

Pseudopodia are ectoplasmic projections which progressively increase in size, frequently becoming digitiform, within which granular endoplasm is projected.

Flagella are long delicate filaments, usually few are present, used by the flagellates for locomotion.

Cilia are short, usually numerous, filaments arising from the ectoplasm.

A **peristome** is a pit or depression in the ectoplasm, which leads to a cavity called the **cytostome** or mouth. This leads to a **cytopharynx** or esophagus.

Vegetative forms of protozoa are called **trophozoites.** The ectoplasm condenses and becomes more resistant, forming the wall of **cysts,** which are resistant forms assumed by the trophozoites in response to unfavorable environmental conditions or as a part of the life cycle.

Chromidia and **chromatoid bodies** are nutritional inclusions in the endoplasm.

The **nucleus** is the fundamental structure of the cell, which participates in the process of cellular multiplication. Not only does it maintain the life of the protozoa, but it is important in the process of reproduction. In many protozoa it is the essential element in morphologic diagnostic differentiation of otherwise similar genera and species, as in the case of amebae of the human intestine. Within the nucleus is a granule or a **chromatin mass.** It is of variable size and is situated excentrically. This **karyosome,** as it is called, stains intensely with iron-hematoxylin. **Chromatin granules,** smaller than the karyosome, are found deposited at the points of intersection of the **linin filaments** which are radial filaments extending in the direction of the **nuclear membrane** and interlacing to form the **linin net** which is impregnated with nuclear fluid.

The flagellates contain a **macronucleus,** or true nucleus, and a small nucleus or kinetoplast or micronucleus, composed of a spherical, ovoid, or bacillary body, called the **parabasal body.** Another smaller, spherical or ovoid body, called the **blepharoplast,** is near the parabasal body, and from this blepharoplast rises the **flagellum,** the initial portion of which is called the **axoneme.**

Classification of Protozoa

There are two subphyla in the phylum Protozoa, Plasmodroma and Ciliophora. Plasmodroma are divided into the classes Rhizopoda, Mastigophora, Sporozoa, Cnidosporidia; Ciliophora into Ciliata and Suctoria. The class Rhizopoda contains five orders, Amoebida, Heliozoa, Mycetozoa, Radiolaria, and Foraminifera. The Mastigophora are the flagellates. The Sporozoa include malarial parasites and coccidia. Of the Ciliophora, only the class Ciliata is of interest in human parasitology.

Order **AMOEBIDA** Calkins 1902

These are Rhizopoda composed of a cytoplasm with a shell or skeletal structure, moving by means of thick, temporary pseudopodia which may appear from any part of the body surface; usually one nucleus, sometimes two, or many. Cytoplasm is usually differentiated into ectoplasm and endoplasm. All the parasitic amebae of the digestive tract of man and animals are included in this order.

The order Amoebida contains the following four families: Amoebidae, Dimastigamoebidae, Rhizomastigidae, and Paramoebidae, with the following differential characteristics:

ORDER			FAMILY
AMOEBIDA	Incapable of forming flagella		Amoebidae
	Form flagella	In adult stage may form two or more flagella	Dimastigamoebidae
		Provided with only one flagellum during the greater part of their free-living existence	Rhizomastigidae
	In addition to a principal nucleus, possess an accessory body (Nebenkörper) which is divided with the nucleus during reproduction		Paramoebidae

Only the family Amoebidae is of interest for our purpose.

Family **AMOEBIDAE** Bronn 1859

This family includes free-living amebae the majority of which parasitize the digestive tract of man and lower animals. These do not have flagella at any period of their existence.

The family Amoebidae has been divided into well-defined parasitic groups, such as the genera Endamoeba, Endolimax, Iodamoeba, and Dientamoeba, and also into free-living groups, not well-defined as yet. Many of the "small" type, formerly all included under the *Amoeba limax,* have since been differentiated into definite genera such as Hartmanella, Vahlkampfia, Sappinia. Most of the "large" varieties are retained in the genus Amoeba until a better understanding of their morphologic and biologic characteristics are understood. The following chart gives a summary of the differential characteristics of the genera of the family Amoebidae.

The first five of these genera include free-living species, with trophozoites usually showing a contractile vacuole in the cytoplasm. They are not of interest in this text. The last four genera, however, Endamoeba, Endolimax,

FAMILY					GENUS
AMOEBIDAE	Trophozoites usually with contractile vacuole in the cytoplasm. Free-living	Small body		Usually, only one contractile vacuole. One nucleus	Amoeba
				Contractile vacuoles have not been observed with certainty; several or many nuclei	Pelomyxa
		Large body		Vesicular nucleus; large central karyosome; numerous chromatin granules in the periphery	Hartmannella
				Nucleus with large karyosome and peripheral chromatin; mononucleated cysts	Vahlkampfia
				Two nuclei closely associated	Sappinia
	Trophozoites without contractile vacuole in cytoplasm. Parasites	One nucleus	Provided with peripheral chromatin	Small central or excentric karyosome	Endamoeba
			Not provided with peripheral chromatin	Voluminous karyosome, usually central, at times excentric; trophozoites small; cysts usually oval, multinucleated; glycogen vacuole usually absent	Endolimax
				Voluminous karyosome, central, spherical, surrounded by a layer of achromatic granules; trophozoites are of medium size; cysts generally spherical or irregular, almost always mononucleated, sometimes binucleated; glycogen vacuole usually present	Iodamoeba
		Generally two nuclei		Not provided with peripheral chromatin; central granular karyosome; small trophozoites; cysts unknown	Dientamoeba

Iodamoeba, and Dientamoeba, include all the parasitic species of the digestive tract of man and are consequently of great importance (see Fig. 448). The species *Endamoeba histolytica, E. coli,* and *E. gingivalis; Endolimax nana; Iodamoeba butschlii;* and *Dientamoeba fragilis* are of interest. The characteristics of Endamoeba are described on the following pages.

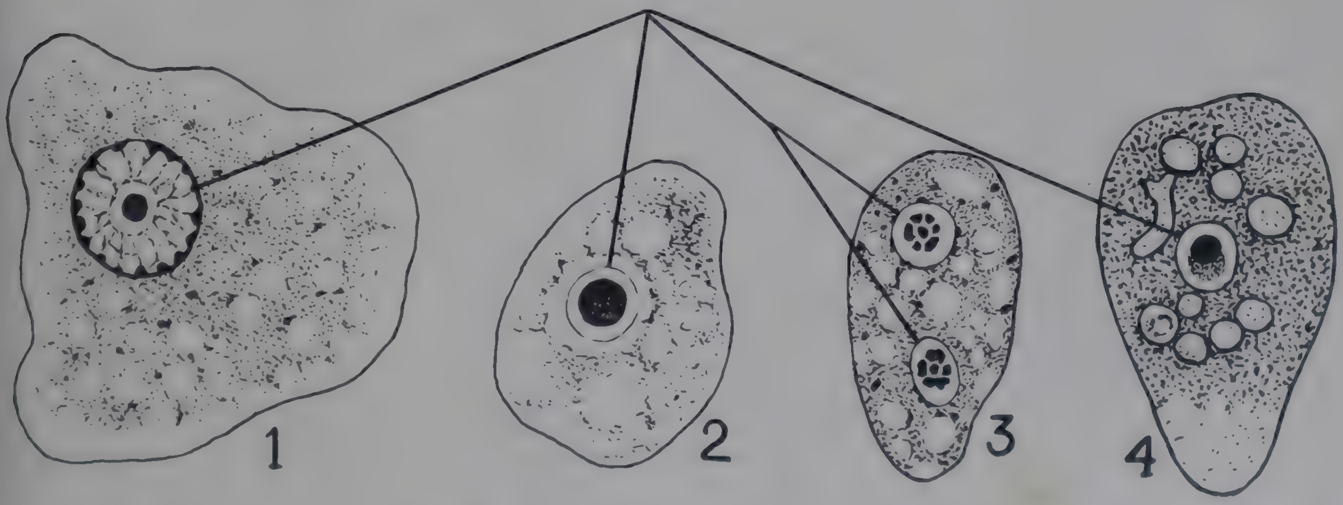

Fig. 448.—Genera of amebae. Nuclear characteristics; partly according to Brumpt and Dobell. *1,* Genus *Endamoeba:* small central or excentric karyosome and a denticulated layer of peripheral chromatin; *2,* genus *Endolimax:* voluminous karyosome, delicate nuclear membrane without peripheral chromatin; *3,* genus *Dientamoeba:* usually two nuclei with a delicate nuclear membrane without peripheral chromatin, and numerous karyosomes in rosette formation; *4, Iodamoeba* (*Pseudolimax*): voluminous central spherical karyosome surrounded by a layer of achromatic granules, crescent-shaped in the cysts, and a delicate nuclear membrane without peripheral chromatin. (Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Genus **Endamoeba** Leidy 1879

Characteristics.—Amoebidae with vesicular nucleus, fine but visible nuclear membrane, karyosome relatively small and situated in the center or near the center of the nucleus. Varying number of peripheral chromatin granules free or adherent to the inner surface of the nuclear membrane. Parasites of man, lower mammals, and invertebrates.

Nomenclature.—Some authors consider the genus Entamoeba as distinct from Endamoeba and include, in the former, the species *histolytica, coli,* and *gingivalis* which are all found in the digestive tract of man. Others believe that Entamoeba and Endamoeba are the same and have thus adopted the generic name Endamoeba since it is the older generic term; they use Entamoeba as a synonym. Using this last nomenclature the three species of this genus which parasitize the digestive tract of man are: *Endamoeba histolytica, Endamoeba coli,* and *Endamoeba gingivalis.*

Endamoeba Histolytica

(Schaudinn 1903) Hickson 1909

Endamoeba histolytica, from the point of view of frequency and of pathogenicity, is the most important of the protozoa which infect the digestive tract of man.

Living Endamoeba Histolytica in Fresh Unstained Preparations.—

Trophozoite.—The living trophozoite of *Endamoeba histolytica,* observed in unstained preparations, appears as a more or less rounded, colorless, refractive organism, 15 to 60 microns in diameter with an average size of 18 to 25 microns. The existence of large and small strains, producing large and small cysts, respectively, has been accepted since 1919 (Dobell). The trophozoite shows a hyaline ectoplasm and a finely granular endoplasm, usually well differentiated, especially when the protozoan is moving about. As a general rule, the nucleus is not observable in a fresh specimen, although at times it can be seen in the form of a ring of small refractive granules within the cytoplasm. In fresh feces the trophozoite contains neither bacteria nor nutritional vacuoles, but these may be present in the endoplasm of dead and degenerated forms. The presence of red blood cells in the endoplasm is almost pathognomonic in the differential diagnosis of this species since *E. coli* shows red blood cells in the endoplasm of its trophozoites only very exceptionally. In such cases the species might be confused with *E. histolytica.* The *E. histolytica* has a marked predilection for red blood cells.

Pseudopodia may be long and narrow (digitiform) or short and wide. They project rapidly. They are extensions of hyaline ectoplasm within which the granular endoplasm is thrown, so that motion results. This motion is so active in fresh feces that there is no clear differentiation between ectoplasm and endoplasm; the pseudopodia seem to be projections of total cytoplasm. As the feces cool, the movement of the ameba becomes slower and there may be seen, first, the projection of the hyaline ectoplasm which forms the pseudopodia, and second the projection of granular endoplasm into the pseudopodia. Later the ameba loses its motility or motility is very slow, until the protozoan dies and becomes rounded.

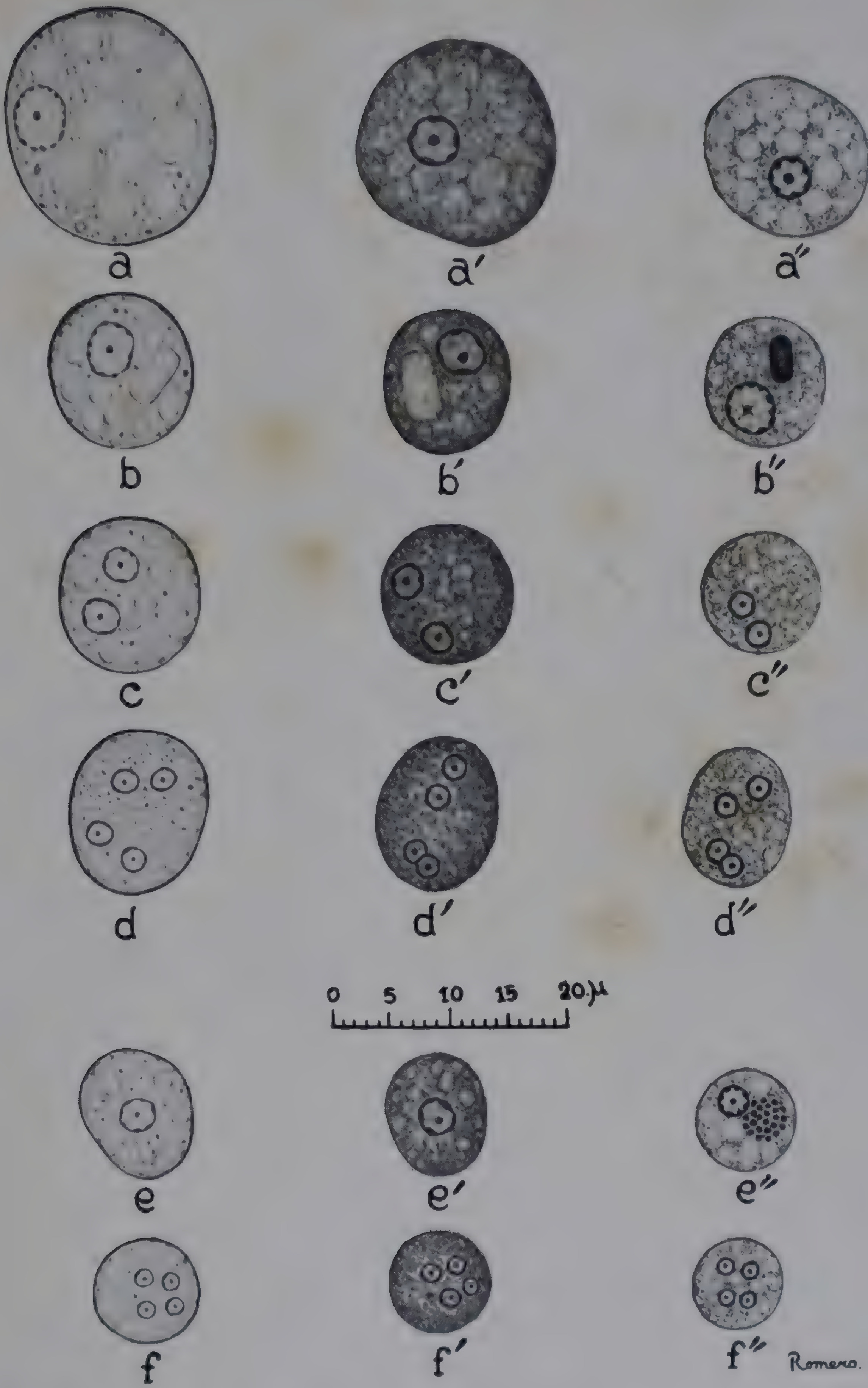

Fig. 449.—*Endamoeba histolytica*: *a* to *f*, fresh specimen; *a′* to *f′* stained with Lugol solution; *a″* to *f″* stained with iron-hematoxylin. *a*,*a′*,*a″*, Trophozoites. Note the peripheral nuclear chromatin, ring-shaped, denticulated on the inner edge, and the small central karyosome and cytoplasm without bacteria. *b*,*b′*,*b″*, Immature uninucleate cysts with a thick chromatoid body in the cytoplasm. *c*,*c′*,*c″*, Immature binucleate cysts. *d*,*d′*,*d″*, Mature quadrinucleate cysts. *e*,*e′*,*e″*, Small trophozoites and precystic forms, with a Sphaerita in the cytoplasm of *e″*. *f*,*f′*,*f″*, Small mature, quadrinucleate cysts. (Original, Kourí.)

Precysts.—In order to change to the precystic form, the ameba becomes immobilized, rounded, and reduced in size, expelling its nutritional contents. Precysts appear as colorless, refractive bodies, round or slightly ovoid. Though they do not present locomotion, they may, nevertheless, slowly project short, wide pseudopodia, which are as slowly withdrawn. The nucleus, when visible, may appear as a series of yellow-refractive granules, distributed in a circle or in the form of a rounded mass, yellow and refractive when viewed by artificial light. Oval or rod-shaped bodies are observed in the cytoplasm. These rods have rounded ends; they are of hyaline appearance similar to chromatoid, crystalloid bodies, chromidia, or rods frequently observed in the younger cyst forms of this species. A cyst wall is lacking.

Cysts.—The trophozoite and the precyst form have only one nucleus, while mature cysts have four and, exceptionally, eight nuclei. Consequently there are intermediate stages between the precyst form and the mature cyst, known as immature cysts, which have two or three nuclei. It is necessary to recognize these in practice.

Cysts appear as round, hyaline bodies, with a cyst membrane which is more refractive than the rest of the cytoplasm. Its dimensions vary widely from small types which produce cysts 3.5 to 12 microns in diameter, to large types which produce cysts 12 to 20 microns in diameter; they may range from 3.5 to 20 microns in diameter.

The nucleus of the cyst has the same appearance as that of the trophozoite. In newly formed cysts, only one nucleus is seen. After division, this nucleus forms two, and by the second division, four nuclei. This number is characteristic of mature cysts and predominates in specimens obtained from fecal matter.

In fresh specimens the nuclei are barely perceptible, the cytoplasm appears colorless and refractive, free of all nutritional inclusions, and in young or immature cysts glycogen vacuoles may be observed. When stained with Lugol solution, cysts are yellow, the cytoplasm loses its refractivity and appears finely granulated, nuclei stand out clearly, and the glycogen contained in the vacuoles stains a mahogany red color. The glycogen vacuole attains its maximum size in uninucleated cysts. These structural characteristics of cysts, trophozoites, and precystic forms are easily seen by use of the oil immersion objective, provided that the optimum amount of light is used.

Fig. 450.—*Endamoeba histolytica* stained with iron-hematoxylin. *1* to *21*, Trophozoites, precystic forms, immature cysts, and mature cysts, in human fecal material in six different cases of intestinal amebiasis (Kourí, Basnuevo, and Sotolongo). *22* to *25*, Trophozoites in pus from a case of amebic hepatic abscess (Case of Kourí and Basnuevo). *1*, Trophozoite with cytoplasm without inclusions; *2*, trophozoite with erythrocyte in the endoplasm; *3*, small trophozoite; *4*, uninucleate cyst with nucleus in activity, pushed back by a very large glycogen vacuole containing a small chromatoid body; *5*, uninucleate cyst with a thick chromatoid body; *5′*, with three chromatoid bodies; *6*, binucleate cyst with two chromatoid masses; *7*, smaller binucleate cyst with three chromatoid bodies; *8*, binucleate cyst without chromatoid bodies; *9*, uninucleate cyst with nucleus in activity, glycogen vacuole, and two chromatoid bodies; *10*, binucleate cyst with one chromatoid body; *11*, mature, quadrinucleate cyst with nuclei at rest, and without chromatoid bodies; *12* to *12″*, small trophozoite: a Sphaerita is seen in the cytoplasm of *12″*; *13* to *19*, uninucleate cysts, without chromatoid bodies and thick wall in *13* and *13′*; with three chromatoid bodies in *14* and *15*; with two Sphaeritas in *16*; with very wide glycogen vacuole in *17*, *18*, and *19*, with numerous chromatoid bodies in *17*; *20*, small binucleate cyst without chromatoid bodies; *21*, mature quadrinucleate cyst with one chromatoid body; *22*, observe the hyaline ectoplasm, very abundant in this typical specimen, with one erythrocyte in the process of being digested and without bacteria in the cytoplasm; in specimen *23*, there are three erythrocytes; in *24* there is one erythrocyte in the endoplasm; and *25*, projecting hyaline pseudopodia. (Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

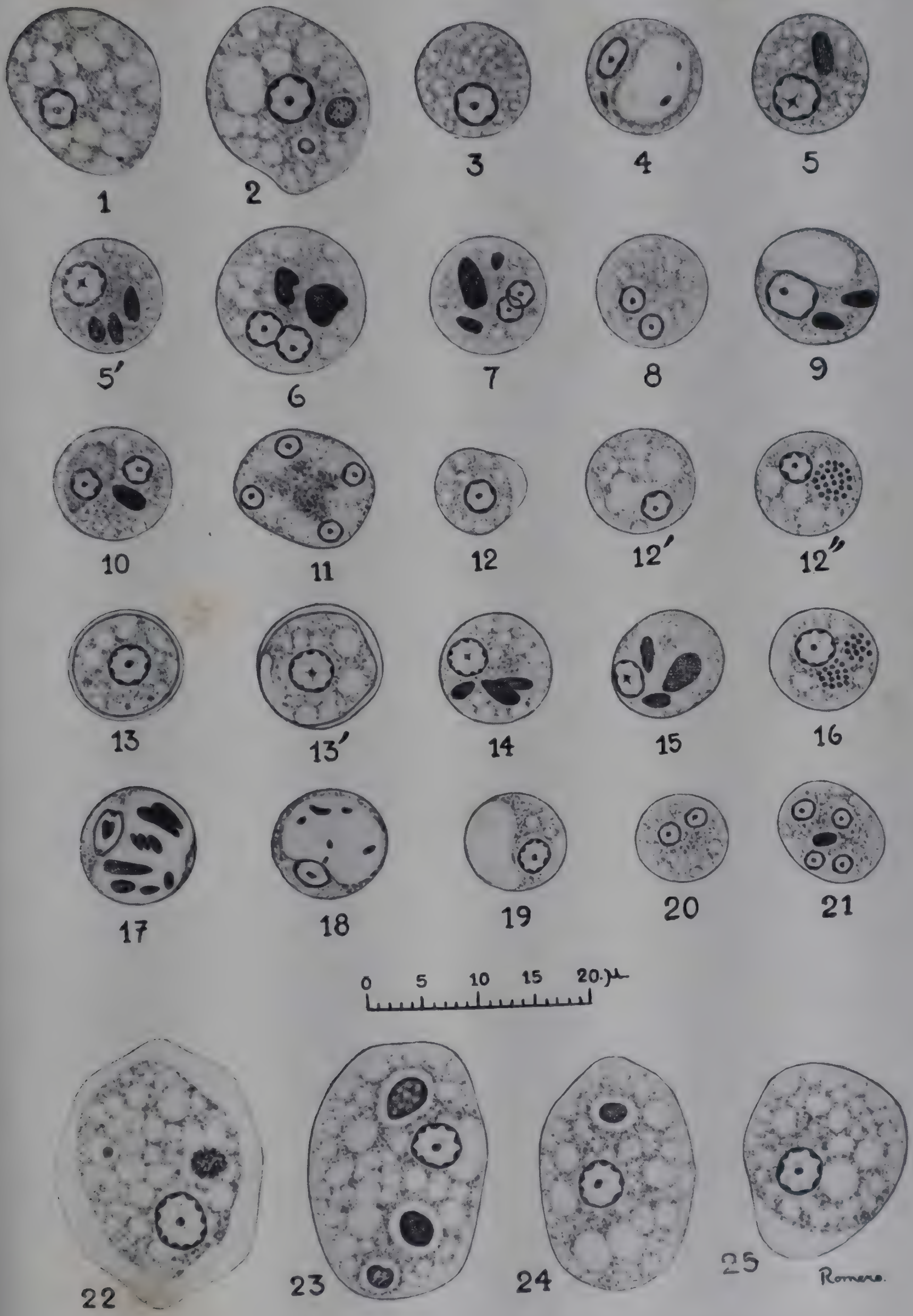
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Fig. 450.—*Endamoeba histolytica.* (*For legend see opposite page.*)

In immature cysts, ovoid or rod-shaped bodies are observed; these are called *chromatoid bodies*. The nature of these bodies has not been definitely determined. They stain a deep black with iron-hematoxylin. They usually disappear in older, mature cysts.

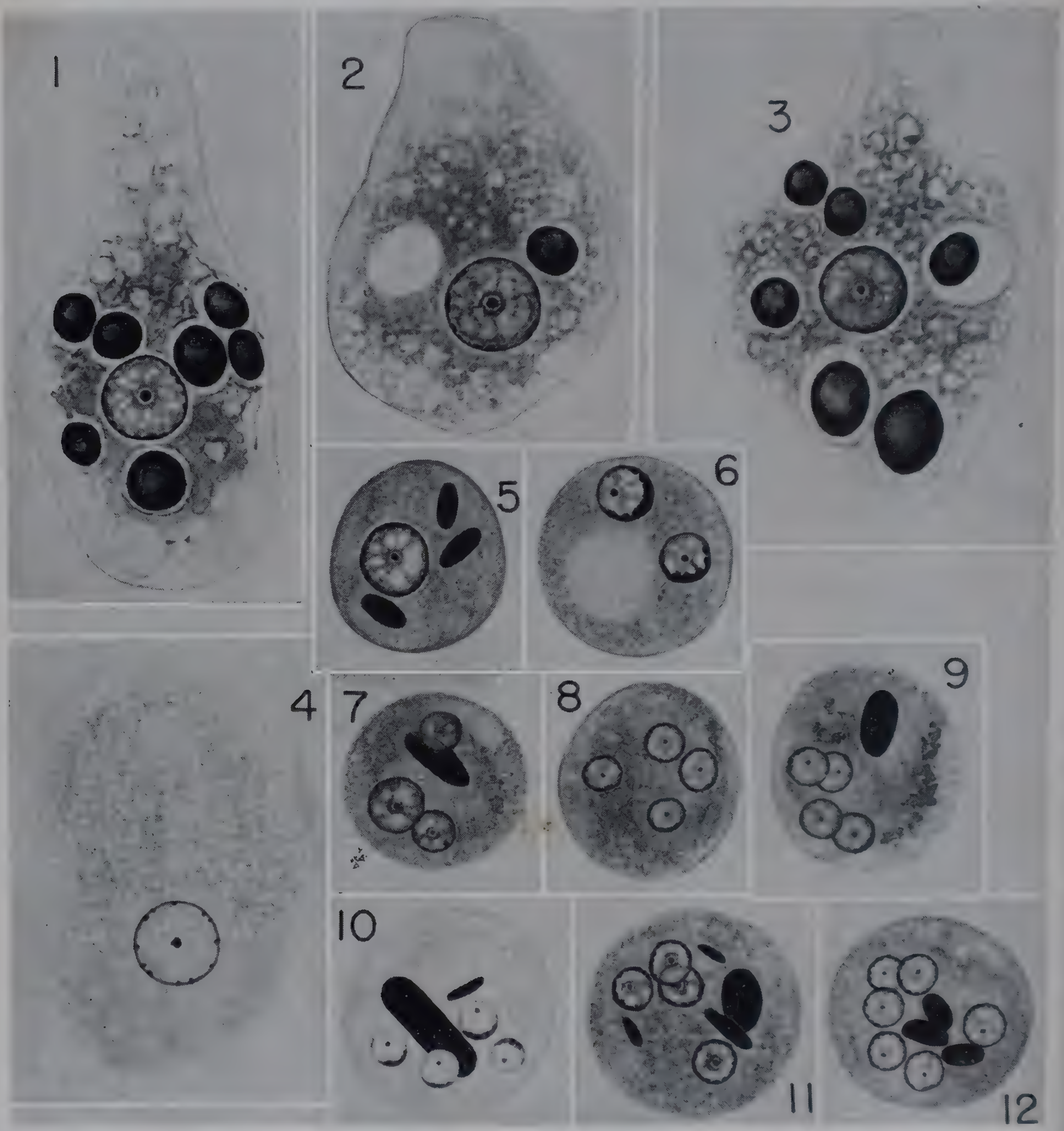

Fig. 451.—*Endamoeba histolytica.* *1* to *3*, Trophozoites from a single case, showing ingested red blood cells; *4*, trophozoite from another case showing delicate cytoplasm and typical nucelus; *5* to *12*, various cysts, with and without chromatoid bodies, showing 1, 2, 3, 4, and 6 nuclei of varying character. Chromatoid bodies are commonly present in young cysts, absent in older cysts. In exceptional cases, 8 nuclei are present. (Courtesy Winthrop-Stearns, Inc., copyright, 1946.)

A few points of difference between a nucleus in repose and a nucleus in the state of activity must be known so that a mature cyst can be differentiated from an immature cyst when the number of nuclei is such as to raise doubts about a differential diagnosis between the *histolytica* and *coli* species. The nucleus at rest is spherical and smaller than the nucleus in activity, which, in addition to being larger, may also be spherical. It is more frequently oval, elliptical, rather elongated, and infrequently fusiform according to the degree of karyokinetic activity at the time of observation. If the cyst is small, and the four nuclei are small and of equal size, it is almost certainly a mature four-nucleated cyst of Endamoeba, species *histolytica;* if the four nuclei are unequal in size and shape, it is probably an immature cyst which, upon maturing, will have eight nuclei, or, less frequently, a multiple of this number, and is almost certainly a cyst of *Endamoeba coli.*

Metacysts.—The cyst of *E. histolytica,* after reaching the lower portion of the ileum, de-encysts and releases an active trophozoite with four nuclei. This trophozoite divides into four trophozoites, one for each nucleus; these grow and invade the intestinal wall to continue their development. These four amebae which result from the de-encystment are called *metacystic trophozoites;* according to Dobell's experiments in vitro, there may be eight.

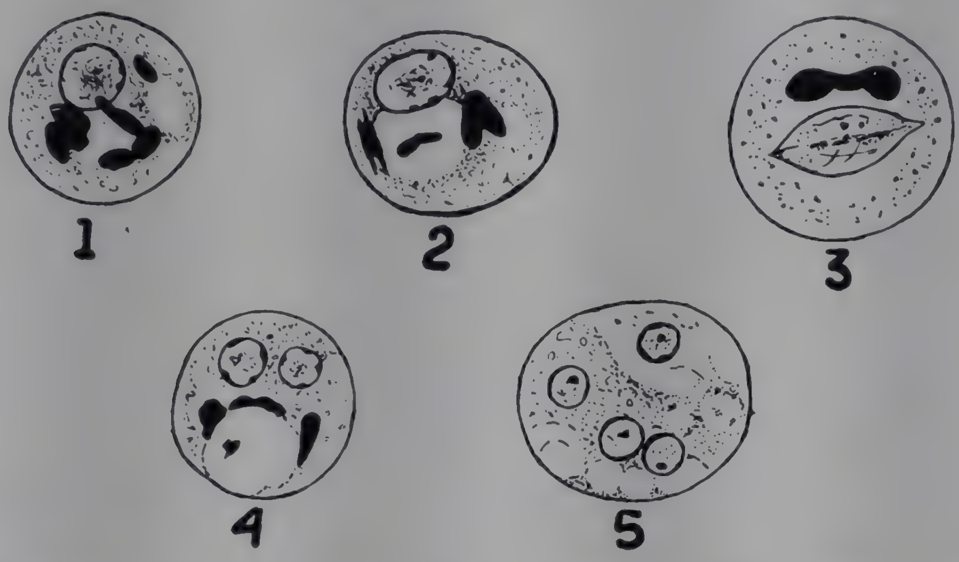

Fig. 452.—*Endamoeba histolytica.* *1* and *2,* Uninucleate cysts with glycogen vacuole and chromatoid bodies; *3,* nuclear karyokinesis in a uninucleate cyst with chromatoid bodies; *4,* binucleate cyst with glycogen vacuole and chromatoid bodies; *5,* mature quadrinucleate cysts. (Brumpt in Kourí and Basnuevo; Lecciones de Parasitología y Medicina Tropical.)

Endamoeba Histolytica Stained With Iron-Hematoxylin.—In well-stained preparations, the cytoplasm of the trophozoite stains grayish or violet and is granular in appearance, with an alveolar and spongy structure. If amebae are at their maximum activity, as in fresh feces, the cytoplasm does not contain bacteria, but red blood cells may be observed. The nucleus is an intense black, and stands out clearly in contrast to the gray or pale violet color of the cytoplasm. The **nuclear elements** are the nuclear membrane, chromatin granules on the inner surface of this membrane in the form of a cogged wheel, and the small karyosome, usually central, but at times slightly excentric, all stained an intense black. Sometimes there can be observed traces of a linin net, in the form of thin fibrils, sometimes radiating from the center to the periphery.

Cysts.—It is relatively easier to identify the cysts of *E. histolytica* in fresh preparations in moist Lugol-stained slides if the high dry objective is used with the 10× or 12× ocular. Staining, however, has certain advantages. It shows clearly the structural characteristics of protozoa, even in cases in which these characteristics might not have been observed in examination of the living specimen and in moist specimens stained by Lugol's. In preparations stained by iron-hematoxylin, the cysts show characteristic morphology and structure. They are spherical, or may be oval, and rarely irregular. The wall does not stain with hematoxylin, but appears as a colorless halo around the cyst. The cytoplasm stains a grayish-blue, while the nucleus and the chromatoid bodies are of a contrasting intense black color. The number of nuclei in mature cysts is usually 4, but some cysts have been observed with 6 or 8 nuclei.

De-encystment of Endamoeba Histolytica.—De-encystment of ingested cysts takes place in the distal portion of the ileum. This permits the young amebae to invade the ileocecal valve and to colonize in its mucosa. It is possible, however, that de-encystment may take place in the caput coli or in the ascending colon.

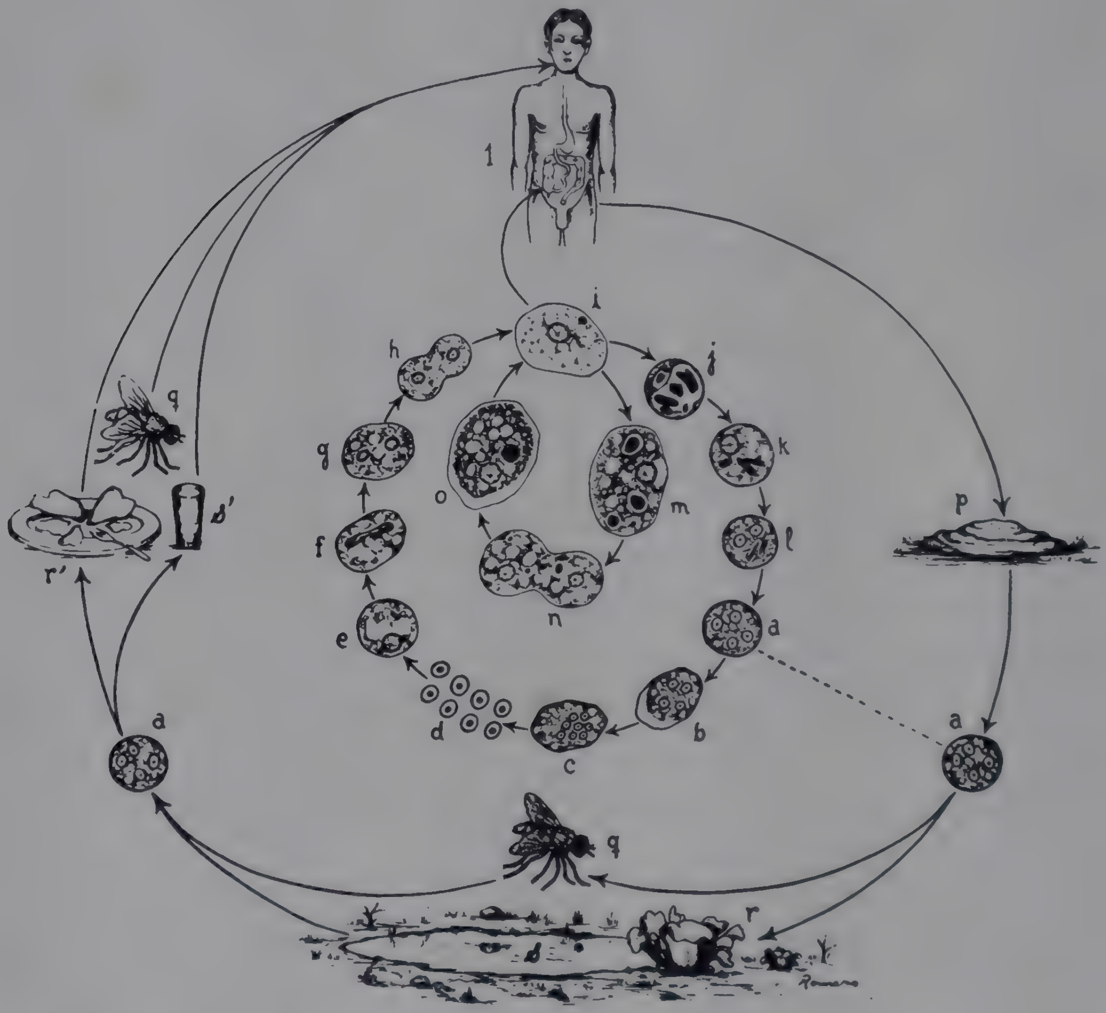

Fig. 453.—Life cycle of *Endamoeba histolytica.* The infective form, the mature cyst (*a*), is passed in the feces (*b*), is disseminated in nature, and contaminates vegetables and water (*r, s*), food for man (*r', s'*); flies (*q*); hands of patients and healthy and convalescent carriers. If the mature cyst (*a*) is ingested by man (*1*), in the intestines the cystic cover is dissolved and a quadrinucleate ameba is liberated (*b*) which, by nuclear division, is converted into an ameba with eight nuclei (*d*). Each develops into a vegetative ameba (*e*) which lives in the lumen of the intestine and reproduces repeatedly (*f,g,h,i,*) by karyokinetic division. In diarrheal or soft feces the vegetative forms (*e,f,g,h,i,*) appear; in hard or pasty stools the precystic forms are found. The immature cyst (*j,k,l*) and the mature cyst (*a*) are the infective forms. Normally the life cycle of *Endamoeba histolytica* is represented in the outer circle, *a* through *l*, without producing definite clinical symptoms. But when the host lacks resistance (*1*) dysenteric syndrome or acute inflammation of the intestines, or a hepatitis develops, and the histolytic forms of amebae (*m,n,o*) develop and bore through the tissue and produce the characteristic lesions of dysenteric amebiasis and its complications. (After Kourí and Basnuevo.)

Resistance of Cysts of Endamoeba Histolytica.—In a cool, moist place, the cysts of *E. histolytica* survive more than 12 days in the feces. In water, the period of survival depends upon the number of bacteria present, and upon the temperature of the water. Cysts survive from 9 to 30 days in indirect proportion to the number of organisms and the temperature. They have little resistance to desiccation and to higher temperatures.

Cysts resist for 15 minutes a 5 per cent solution of cresol, for 30 minutes a 1:2,500 solution of bichloride of mercury, 5 per cent Formalin, and 1 per cent phenol.

Cysts may be ingested by flies and eliminated with their excreta in a viable condition as long as 48 hours after ingestion.

Infective Forms of Cysts.—Mature or quadrinucleate cysts constitute the infective forms. These enter the human organism by way of the mouth, passing undamaged through the stomach, thence to the small intensine. Here the cyst wall softens and breaks, due to the action of digestive ferments, and a quadrinucleate ameba is released. The quadrinucleate ameba undergoes two cytoplasmic divisions without further nuclear division, giving rise to four active amebae. Occasionally nuclear division may also take place; this results in the production of eight very small amebae.

Life Cycle.—The metacystic amebae migrate into the large intestine and establish themselves in the walls, especially in the ileocecal region and in the first part of the ascending colon, as well as in the rectosigmoid. At these sites, at autopsy, there is seen a predominance of lesions caused by *E. histolytica*. The infection is very persistent; it may last throughout the life of the host.

While in the intestinal mucosa, the amebae multiply repeatedly by binary fission. For reasons as yet unexplained, some of these amebae that remain in the intestine expel their nutritional material, undergo reduction in size, possibly by division, and become rounded, constituting the so-called *precystic* forms. These precystic forms are later enveloped by a resistant membrane, forming the uninucleate cysts. When they mature, they are transformed into the quadrinucleate, infective forms. These forms, if ingested by the proper host, begin again the life cycle just described.

Mechanism of Transmission

Source of Infection.—*E. histolytica* is usually transmitted directly from person to person by food handlers, housekeepers, nurses, servants, etc., or by food or drink contaminated by excreta of parasitized persons. Although Faust succeeded in infecting young cats and dogs by feeding them trophozoites, nevertheless in nature the cyst is the usual infecting form. Since the so-called *normal carriers,* or better, *carriers without symptoms,* or with light symptoms without diarrhea or dysentery, regularly eliminate amebae in cyst form, while the diarrheal and dysenteric subjects eliminate the vegetative form of the parasite, the trophozoite, it is the mis-named *healthy carriers* who constitute the principal source of dissemination and contagion of *E. histolytica.*

Contamination of Food and Drinking Supplies Due to Inadequate Inspection of Water.—In cities where the water system is in bad condition, water pipes may permit contamination of drinking water. Installation of filters, chlorination of the water, and above all, boiling the water are indispensable in preventing infection. As an example of contamination by drinking water, we cite the classic example of amebic dysentery in Chicago, where 1,050 persons were infected by contaminated water or food used in two hotels in that city, and which resulted in 70 deaths.

In rural towns which have no centralized water works, drinking water from rivers, brooks, springs, wells, etc., is readily contaminated by human excrement which is often deposited upon the surface of the ground. When these rural towns are visited by whole families, armies, explorers, etc., who may find it necessary to drink from such sources, these people are exposed to infection by *E. histolytica* and by other parasites prevalent to the region which have the same mechanism of penetration.

Handling of Food by Infected Individuals.—In modern cities which enjoy the best general hygienic conditions, particularly a pure source of water supply, adequate disposal of human excrement, etc., incidence of amebiasis may still be high because of direct transmission of the organism from person to person. Such people as food handlers, cooks, waiters in public and private service, nursemaids, wet nurses, etc., should show a certificate of health which should include examination for intestinal parasitism. Vigilance should be maintained by periodic examination of their feces. These individuals present a source of danger as disseminators of the disease. They may be carriers without symptoms or with slight symptoms, the principal eliminators of cysts. These individuals may pass unnoticed since they do not present symptoms of amebiasis. It is important to discover these "normal carriers" and submit them to adequate treatment before permitting them to return to their work, especially if they handle food. In this respect, the work of Schoenleber (1940) demonstrating the importance of this method of transmission of *E. histolytica* is very significant. His report concerns the island of Aruba, near the coast of Venezuela, to which both drinking water and food were sent from the United States. Amebiasis was prevalent on this island, reaching 25 per cent of the population with 36 cases per 1000 with amebic colitis. These infections could not have been due to contaminated food or water, since these came from a safe source. It was later verified that the only possible means of contamination was through some 33 per cent of the Chinese "food handlers" who were infected by *E. histolytica*.

Once the handlers were isolated and cured of their amebic infection the total percentage of infected population dropped by 50 per cent in one year and 92 per cent in three years; at the same time incidence of amebic colitis decreased by 86 per cent in the first year and 92 per cent in three years, all other conditions remaining the same.

Excreta of Flies and Other Insects.—This mode of transmission is important in those areas in which the insect is abundant and where food is unprotected from them. If a cyst is deposited by a fly on liquid or solid food, the infection is transferred to the digestive tract of man. This type of transmission occurs particularly in crowded areas such as military camps, recreation camps, or work camps. As an example, Craig reported an epidemic of amebic dysentery in El Paso, Texas, widespread among the troops stationed there. This epidemic was due in all probability to food contaminated by excreta of flies. Faust (1943) cited a similar epidemic in Kuling, China (1921), caused by a plague of flies coming from a near-by region where there was a high percentage of ameba carriers.

Not only excreta, but also the body of the insect, the wings, and especially the feet and proboscis of the fly can transport cysts mechanically from the sources of infection to food.

Use of Human Excreta as Manure.—The custom of utilizing human excreta as manure is a common practice by farmers of the Far East. Due measures should be taken to stop this method of fertilization, since the cysts of *E. histolytica* remain alive for several days in moist fecal media.

Lack of Personal Hygiene, Crowding in Prisons, Nurseries, Asylums, Orphanages, Etc.—In such crowded areas as concentration camps, prisons, military camps, orphanages, schools, nurseries, hospitals, asylums, etc., crowding constitutes a great danger of propagation of *E. histolytica.* This danger is in direct proportion to the lowering of standards of personal hygiene. There is maximum danger when one considers that transmission from person to person is one of the most effective means of propagating amebae.

Physical cleanliness, bathing, frequent washing of the hands, especially after visiting the toilet and before eating, should be advised.

Susceptible Animals.—The following animals have been found infected with *E. histolytica* in their natural environment:

Monkeys, according to the studies of Dobell (1931) and Hegner (1934); mice—Chiang (1925) and Andrews (1934) found this ameba in the intestines of mice; cats; dogs (Faust 1930) in the United States; young rats, guinea pigs, hamsters, hogs in China, according to Kessell (1928).

The following animals have been experimentally infected: kittens, dogs, monkeys, guinea pigs, rats, rabbits, and hogs. Of these, kittens and dogs are most susceptible and suitable for experimental infection.

Distribution

Geographically, *E. histolytica* may be called a cosmopolitan parasite. Every region which keeps statistics on intestinal parasitism has reported such organisms. It is found more frequently in tropical and subtropical regions than in the temperate zones, although it may be frequent in these if sanitary conditions are bad.

Age.—According to Faust (1933) children under 5 years of age are less often infected than are those older than 5, and adults. The ages 26 to 30 years show the highest incidence of infection, the percentage declining after the age of 35 years. Lesser infection of infants is probably due to less exposure to contagion, while the decline in incidence after the age of 35 is probably due to immunity acquired by previous infections.

Pathologic Anatomy of Intestinal Lesions

Intestinal lesions vary from slight superficial erosion of the intestinal mucosa to extensive and deep lesions and remote extraintestinal complications, with formation of abscess in the liver and other viscera as well as lesions and ulcerations in various tissues not connected to the digestive tract.

There are three characteristic aspects of amebic lesions of the intestines: (1) nodular thickening in the vertex of the folds; (2) ulcers with irregular contour and excavated borders; (3) ulcers connected with each other beneath normal mucosa, which is undermined by tubules containing gelatinous and necrotic material, which show trophozoites of *E. histolytica;* (4) in less severe cases of amebic dysentery and in carriers, lesions starting with necrosis of the superficial portions of the mucosa, extending laterally and into the depths of the tissue, producing rounded or oval ulcers with congested and thickened but not undermined borders.

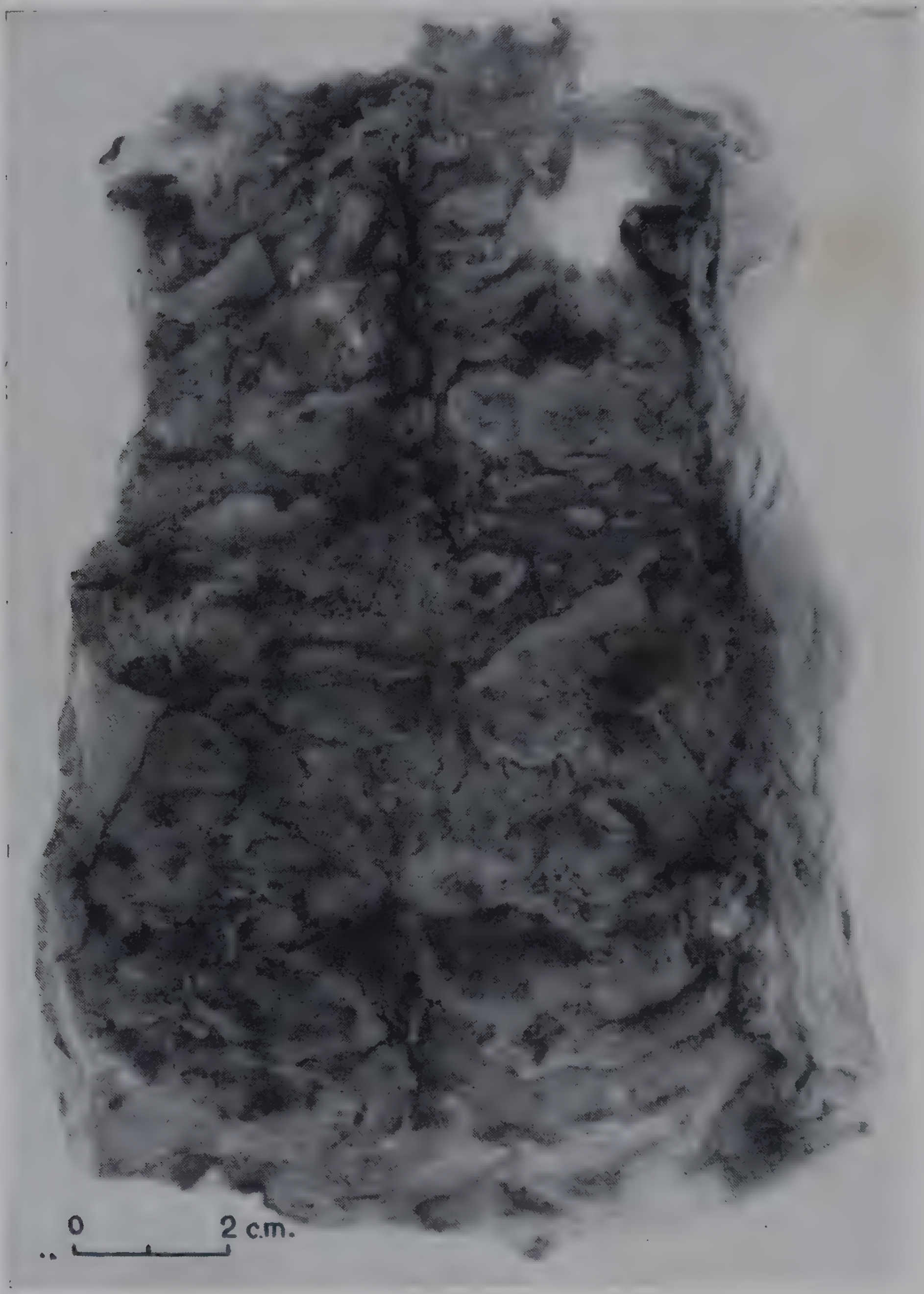

Fig. 454.—Intestinal amebiasis. Macroscopic appearance of the lesion. Note the extent, intensity, and severity of the lesions; ulcerations and necrosis. (Anatomical section No. 52, Finlay, Museum, Institute of Tropical Medicine, University of Havana.) (Original, Kourí.)

Hepatic Lesions

Invasion of the liver is known as amebic hepatitis. It is more than an inflammatory lesion; rather is it a necrotic affair, in multiple or small areas, round or oval, reddish gray, and of diffuse form. The foci coalesce, necrosis is complete, and an abscess is formed, with a cavity filled with necrotic tissue.

definite in contour, with a fibrous wall, generally localized in the right lobe. Occasionally the abscess may be multiple, smaller, and located anywhere in the liver parenchyma.

Other Lesions

The most frequent extraintestinal localization is the hepatic. Other localizations in the order of frequency are: **pulmonary,** primary abscess of the lung; secondary **pleuropulmonary** complications or those following upon rupture of an amebic hepatic abscess which opens into the pleural cavity or directly into the right pulmonary base by pre-established adhesions.

Amebic abscess of the **cerebrum** and amebic invasion of the **skin** are much less frequent than pulmonary amebiasis. Amebiasis of the **urinary and biliary systems** and **duodenal** localization of amebic lesions have also been reported by different authors.

Diagnosis

Sir Phillip Manson-Bahr has emphasized the need for the use of a proctoscope in the diagnosis of amebiasis. He advises the use of the Volkmann spoon with a long handle to obtain scrapings from the rectum. He states that it often succeeds when other methods fail. Manson-Bahr contends that ulcers in the rectum are quite unlike the appearances seen in textbooks: they are almost microscopic in size and protean in character. The acute stage looks like ulcerative colitis. Mucosa is friable and bleeds when swabbed with cotton wool. When scrapings are made from the surface, amebae are found in mosaic-like masses. In the acute stage it is possible to identify the tissue-invading trophozoites of *E. histolytica* moving between the cells, and in the more chronic stages to find cysts and precystic stages. Manson-Bahr does not believe that radiography as a means of diagnosis has much practical value.

Indirect Diagnosis by Means of the Complement Fixation Test

Complement fixation tests, according to Craig, give 90 per cent positive reactions in infections by *E. histolytica*. Bozicevich, Hoyem, and Walston[1] described a complement fixation test for amebiasis which eliminates most of the difficulty previously encountered in this test. Refer to pages 1831 and 1835.

Diagnosis by Means of Stains and Cultures

Staining methods for amebae are found on pages 1820 ff.

Collection of Specimens

The stool must be absolutely fresh. It should be examined within a few minutes after collection. If this basic rule of stool examination is not followed, the amebae disintegrate and sensitive microorganisms either die or are so outgrown by secondary microorganisms that they cannot be easily recovered.

The most suitable portion for examination is mucus. If no mucus is found, samples are taken from the surface and also from the inner portions of the specimen. Postcathartic stool must be examined when detecting carriers of *E. histolytica*. Such stools are collected after the patient has received 1 to 3 teaspoonfuls of sodium or magnesium sulphate in one-half glass of water before breakfast. The samples for examination are collected from the second bowel movement.

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[1] Pub. Health Rep. **61**: 15, 1946; Lab. Digest **10**: Sept., 1946.

D'Antoni (1942) described a method which consists of a cleansing enema about 1 to 1½ hours before sigmoidoscopy. Then an enema with about 2 liters of tepid saline is given. The last part of the returned enema fluid is used for examination. Finally, aspiration samples are taken from the bowel walls through the sigmoidoscope. When a cathartic is given before the enemas, postcathartic stools may also be collected.

Another method for rectal examination is the swab of Hardy and Watt, which consists of a 10 to 12 cm. piece of rubber tubing of 0.18 cm. outside diameter, fitted over a regular throat swab. The throat swab is prepared from a wooden applicator with one end covered by rolling cotton tightly over it. Before use, the end of the applicator is inserted just inside the rubber tube. The end of the tube is lubricated and inserted for a distance of about 5 to 6 cm. into the rectum. The swab is then exposed by withdrawing the tube 2 or 3 cm. The swab is gently rotated, then withdrawn into the tube, and the whole is withdrawn from the patient. The tubing is discarded into a container and sterilized by boiling. The swab is used immediately to make permanent stained slides for examination for intestinal protozoa. Because of the lubricant, the fecal material on the swab cannot be used for saline and iodine-stained suspensions for direct examination.[1]

Endamoeba Coli (Grassi 1879)*

Endamoeba coli is frequently found in nature, lives in the lumen of the large intestine, and feeds on bacteria, food fragments, and protozoa and their cysts. It is somewhat similar to *E. histolytica.* It is usually described as being larger, from 20 to 40 microns.

The **ectoplasm** is much less hyaline and transparent than that of *E. histolytica* and is not well differentiated from the endoplasm. In the **endoplasm** there are a large number of vacuoles containing a variety of nutritional materials—bacteria, yeasts, cysts of other protozoa, etc. The **pseudopodia** are similar in shape to those of *E. histolytica* but frequently they are blunt and are not projected in any definite direction.

The **nucleus** is characterized by peripheral chromatin granules, coarser and more irregular than those of *E. histolytica.* They are more readily distinguishable, due to their refractile character. The karyosome, which is larger and less refractile, is excentrically located.

Precystic forms are about 20 microns and are similar to those of *E. histolytica,* from which they can be differentiated by nuclear characteristics.

The **cysts** are spherical or spheroidal, seldom irregular, and vary from 10 to 30 microns. The membrane which surrounds them presents a well-marked double contour. They are originally uninucleate; through successive division they form two, four, and eight nuclei, eight being considered typical of mature cysts. They contain a large amount of glycogen, which ac-counts for their dark brown color when tinged with iodine. They frequently lack **chromatoid bodies**; when present, these are needle-shaped and sometimes joined together, forming large or small clusters.

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*After Beltrán, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, The C. V. Mosby Co.

[1] O. Felsenfeld, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, The C. V. Mosby Co.

Life Cycle.—The life cycle is similar to that of *E. histolytica* except that this ameba, once established in the large intestine, lives within the lumen and feeds on bacteria and other material without invading the tissue, and is thus considered a commensal.

The **incidence** is high. It has been estimated that about 50 per cent of the entire population of the world harbors *E. coli.*

The mechanism of **transmission** is the same as that of *E. histolytica.*

This species is difficult to cultivate.

The parasite is not easily transmitted to animals.

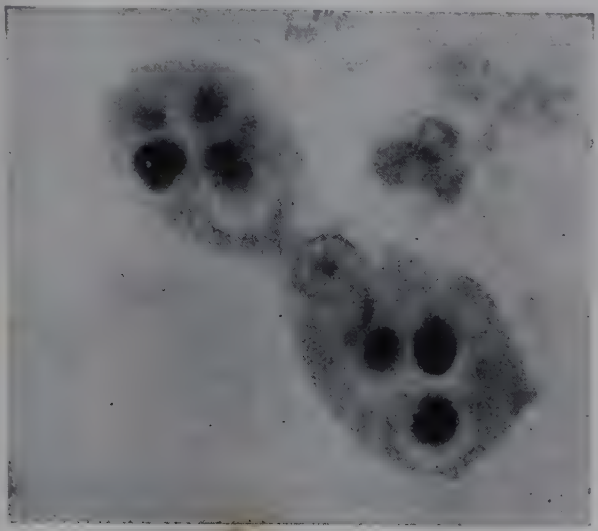

Fig. 455.—Trophozoite of *Endamoeba gingivalis,* stained with iron-hematoxylin. The nucleus is seen in the central portion, the remnants of ingested leukocytes in the food vacuoles. (×3,000.) (Original photomicrograph of E. Beltrán in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Differentiation Between Endamoeba Histolytica and Endamoeba Coli

The cysts and trophozoites of *E. histolytica* are usually smaller than those of *E. coli.* Size, alone, however, is not an absolute means of differentiation, since at times the sizes vary and cysts and trophozoites of *E. histolytica* are rather large.

In normal and vigorous living specimens, the most important means of identification is differentiation of the ectoplasm and endoplasm and the nature of the amebic movements.

The contents of the food vacuoles is very important: *E. histolytica* habitually shows only ingested erythrocytes, or completely lacks food vacuoles, whereas *E. coli* shows abundant food vacuoles containing a variety of material. In degenerated *E. histolytica* are seen vacuoles containing ingested bacteria, and there are some reported cases of *E. coli* containing ingested erythrocytes.

Cysts help differentiate the two species: the size, nature of the membrane, single in *E. histolytica,* double in *E. coli,* and the nature of the chromatoid bodies. Of greater importance is the structure of the nuclei which, as in the trophozoites, offers a more certain identification in stained slides. The number of nuclei is also a means of differentiation, but this is not always reliable. It appears that even though the number of supernucleate cysts of *E. coli* may be large, up to 20 per cent, this is of no particular importance in diagnosis, whereas the number of supernucleate cysts in *E. histolytica* which might cause serious confusion is barely 3 to 4 per cent.

Endamoeba Gingivalis (Gros 1849)

E. gingivalis, although not a true intestinal ameba, since it lives in the mouth, is mentioned here only because of its high incidence; it occurs in about 50 per cent of adults. Morphologically it resembles *E. histolytica* from which it differs in a few slight details of nuclear structure. The food vacuoles contain a variety of materials, such as bacteria and other microorganisms and the remnants of leukocytes.

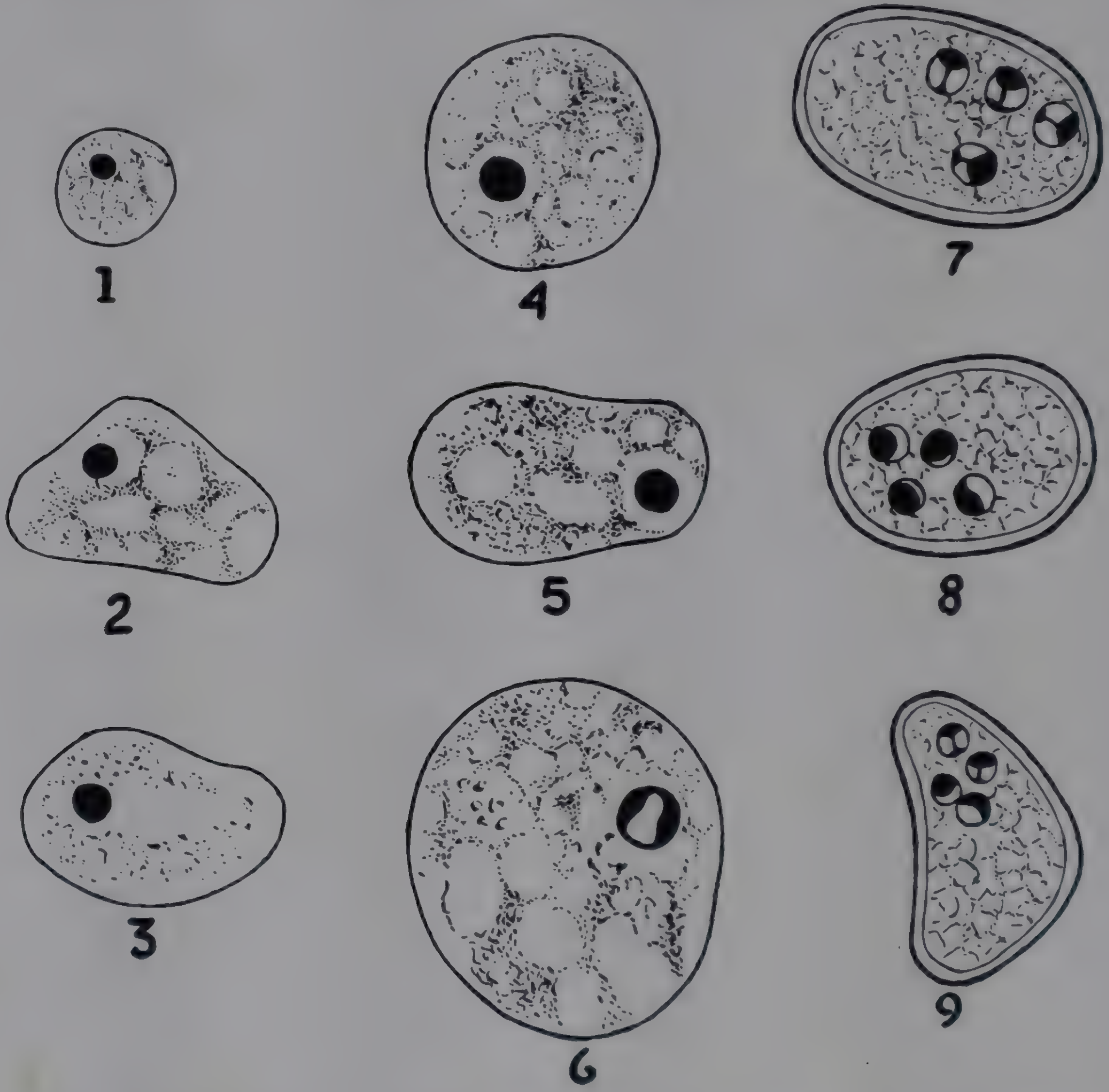

Fig. 456.—*Endolimax nana.* *1* to *6*, Trophozoites of different sizes, after Brumpt. *7* to *9*, Cysts. (After Wenyon and O'Connor in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

This species has no cystic form in its life cycle. It is assumed that transmission from person to person takes place by direct contact, especially through kissing.

Some authors consider it to be of etiological importance in pyorrhea alveolaris, although most contemporary authors do not accept this view.

Endolimax Nana (Weynon and O'Connor 1917)

Endolimax nana is a small ameba, varying from 6 to 12 microns. Differentiation between the ectoplasm and the endoplasm is variable; while perfectly marked in some, in others it is observed only with the greatest difficulty, or not at all. The karyosome is large, irregular, excentric, and without peripheral chromatin. The endoplasm contains abundant food vacuoles containing a variety of material. This fact, in conjunction with the characteristics of their movements, sluggish and in different directions, sometimes causes this species to be confused with small *E. coli*. The nucleus constitutes the most important diagnostic characteristic of the parasite.

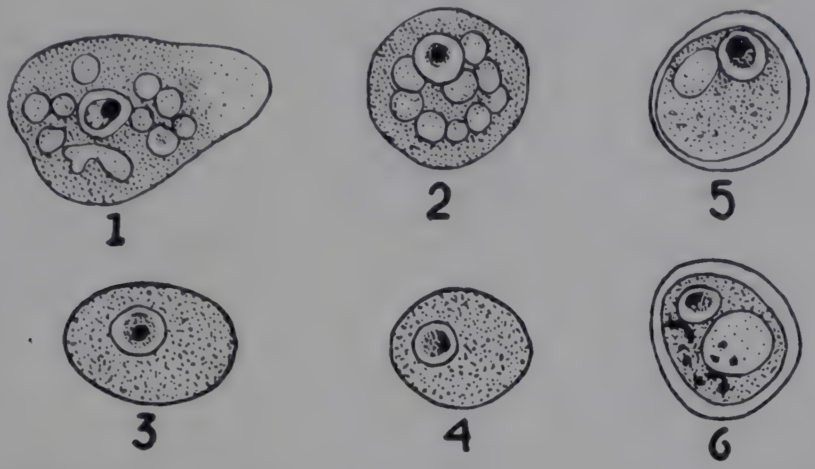

Fig. 457.—*Iodamoeba williamsi.* *1* and *2*, Trophozoites. *3* and *4*, Precystic forms. *5* and *6*, Mature cysts with glycogen vacuoles and a single nucleus. (Original figure of Brumpt in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

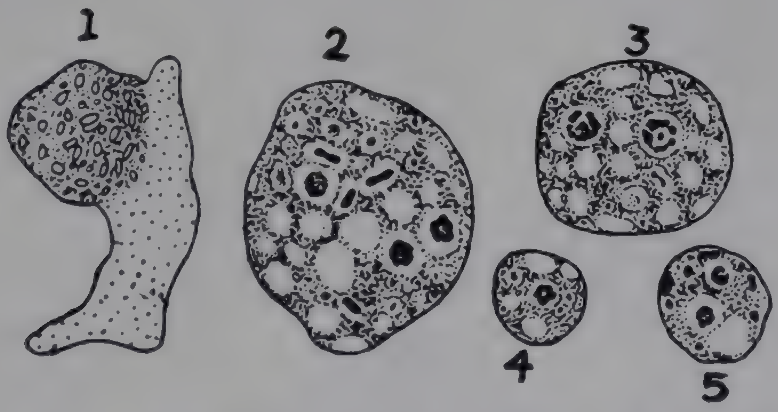

Fig. 458.—*Dientamoeba fragilis.* *1*, Living trophozoite. *2* to *5*, Trophozoites, fixed and stained. (After Jepps and Dobell in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Cysts are ovoid, less frequently spherical, with four nuclei in the mature state. The nuclei have the same characteristics as those found in the trophozoite, but they are barely visible in fresh specimens.

They measure approximately 8 to 10 microns along the longer axis. Young stages frequently show glycogen, distributed diffusely, or concentrated in vacuoles, which sometimes resemble those found in Iodamoeba. Dobell described the occasional presence of filaments which stain black with iron-hematoxylin.

The **life cycle** is similar to that of *E. coli.* It is a simple commensal.

Incidence is about 10 per cent; in Mexico, it is 8 to 32 per cent (Beltrán).

Endolimax nana disappears from the feces after oral administration of pentavalent arsenicals. It is frequently found parasitized by Sphaerita. It has been cultivated in the same media as that used for culture of *E. histolytica.*

Iodamoeba Williamsi (Prowazek 1911)

Synonym: *Iodamoeba bütschlii.*

Iodamoeba williamsi are small organisms, varying from 5 to 20 microns. The **nucleus** has a generally well-defined membrane enclosing a thick, spherical excentric karyosome surrounded by a mass of achromatic granules which, in the cyst, have a semilunar arrangement. The **ectoplasm** is not clearly differentiated from the endoplasm. The **endoplasm** contains numerous nutritional vacuoles with the most varied contents. Movement of the ameba is sluggish, frequently not directional.

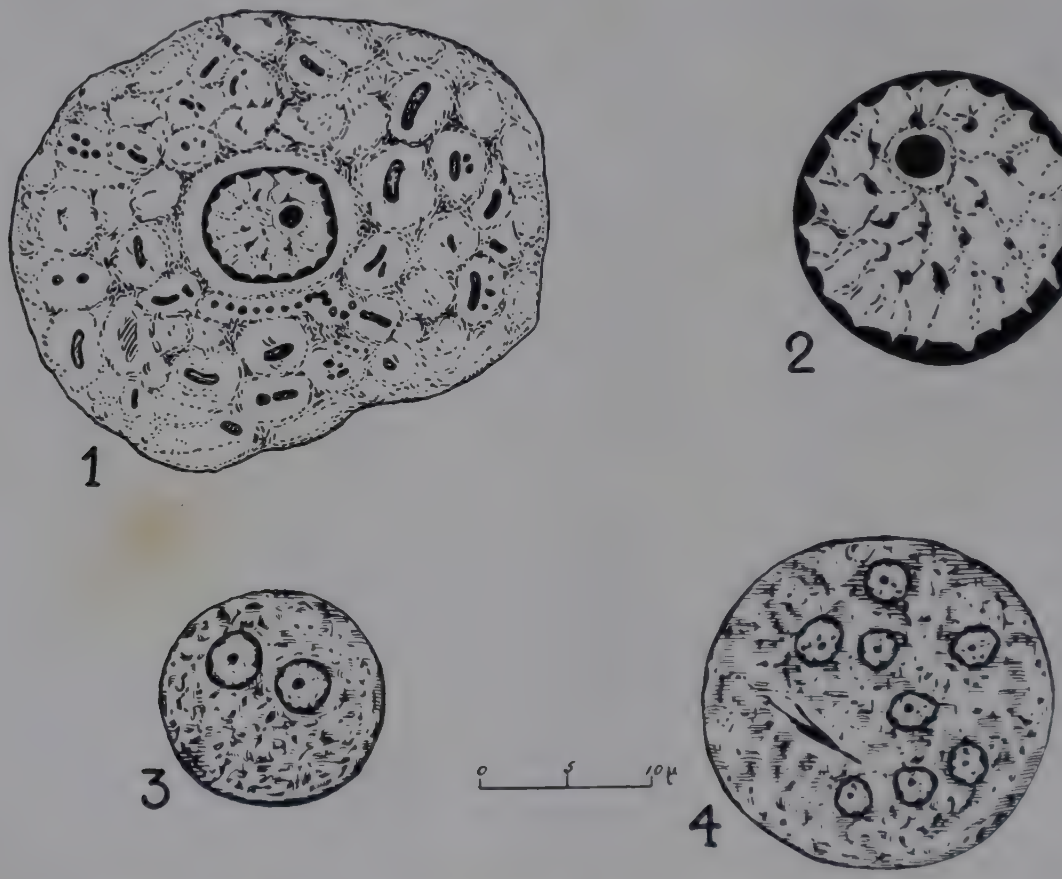

Fig. 459.—*Endamoeba coli.* Iron-hematoxylin stain. *1,* Trophozoite without hyaline ectoplasm, without erythrocytes in the endoplasm, but containing bacteria. Nucleus central with excentric karyosome and thicker than that of *E. histolytica. 2,* Nucleus greatly enlarged to show its characteristics. Thicker denticulated peripheral chromatin, excentric karyosome thicker than in *E. histolytica,* and small chromatic granules disposed in the spaces between the karyosome and the nuclear membrane. *3,* Small binucleate cyst. *4,* Normal size mature cyst with eight nuclei. (After Langeron and Rondeau du Noyer in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Cysts, 5 to 18 microns in diameter, are frequently uninucleate, sometimes binucleate. The nucleus is practically invisible in fresh specimens and barely detectable in preparations treated with iodine. The most notable characteristic is the great irregularity of outline. Another characteristic structure is the **glycogen vacuole** with its definitely limited outline, which occupies a large part of the cyst. In fresh specimens or in specimens stained with hematoxylin, it appears as a clear space, while in specimens stained with iodine it appears as a well-defined, mahogany-colored mass.

It has often been stated that the cysts are found in large numbers in infections due to this species, but that only rarely are the trophozoite forms

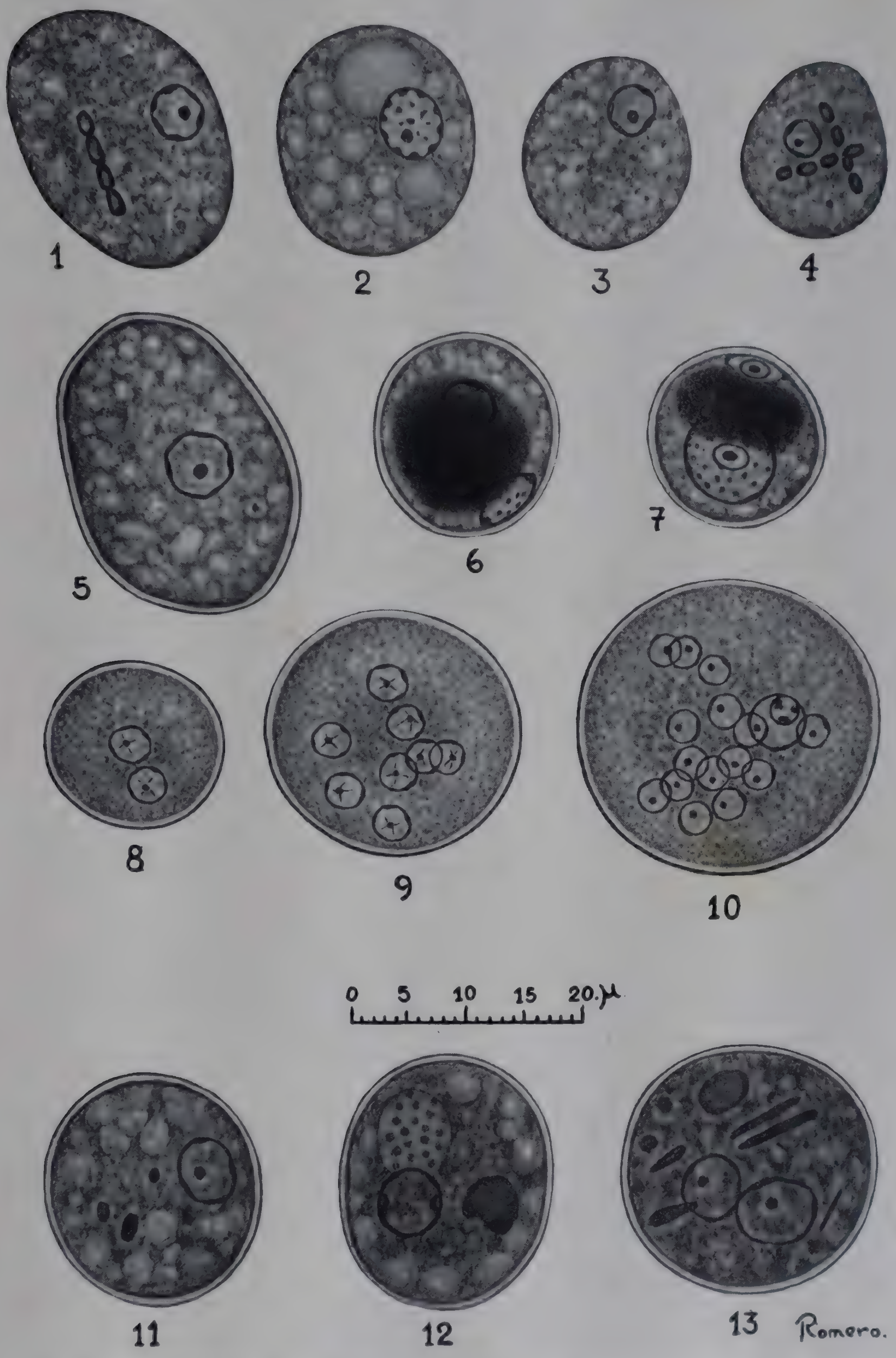

Fig. 460.—*Endamoeba coli.* Stained with Lugol solution. *1* to *4*, Trophozoites. Note bacteria in the cytoplasm of *1* and *4*, excentric karyosome in all four, and chromatic granules between the karyosome and the nuclear membrane in *2*. *5* to *7*, Precystic form and immature cysts with two nuclei and glycogen vacuole in *6* and *7*. *8*, Small immature binucleate cyst. *9*, Mature cyst with eight nuclei, normal size; and *10*, gigantic mature cyst with sixteen nuclei. *11* to *13*, Degenerative forms. (Original of Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical. Drawing by Romero, with 10 × ocular, oil immersion objective.)

found. Beltrán has found the vegetative forms sufficiently abundant in both liquid and formed stools.

The **life cycle** is similar to that of *Endolimax nana.* It is usually considered harmless to man. Incidence is about 7 to 31 per cent (Beltrán). It disappears after oral administration of pentavalent arsenicals. At times it is parasitized by Sphaerita. It can be cultivated in the same media as used for *E. histolytica.*

Dientamoeba Fragilis (Jepps and Dobell 1918)

These parasites are easily destroyed, for which reason the name *fragilis* has been given to this species. They disintegrate readily in fresh specimens and in Lugol preparations. They are very small, 4 to 12 microns. The ectoplasm and endoplasm are clearly differentiated. They project hyaline, transparent pseudopodia which resemble those of *E. histolytica,* although they are not formed with the same rapidity.

The **nucleus** is the most outstanding characteristic. There are often two nuclei, which are vesiculated, with delicate, nuclear membrane, without peripheral chromatin, with central karyosomes formed by dense chromatin granules. **Cysts** are unknown.

The **life cycle** differs from other parasitic amebae of man, with the exception of *E. gingivalis,* in that no cysts are known. Because of this, details of the transmission are still obscure.

Most authors agree that this is the least frequent of the amebae of man. Beltrán has found an incidence of 9 per cent.

Most authors agree that this organism is not pathogenic for man, but this point has not as yet been determined.

Degenerated forms are similar to those of *Blastocystis hominis,* with which it is sometimes confused.

These amebae die readily in an outside environment.

Dientamoeba fragilis has been cultivated in the same media as those used for *E. histolytica.* It is inoculable into cats. It disappears from the feces after ingestion of pentavalent arsenicals.

MASTIGOPHORA

Mastigophora are characterized by having, as organs of locomotion, long vibratory whiplike appendages, called **flagella,** from which is derived the name "flagellates," commonly applied to them.

Certain flagellates live in the blood and the tissues; these are called *hemoflagellates.* Another group lives in the intestine; these are called *intestinal flagellates.* Generally included in this group, although they are not, strictly speaking, intestinal inhabitants, are species found in the mouth and in the vagina.

Polymastiginia, one of the four orders into which this class is divided, is characterized by three to eight flagella, with the exception of the family Polymonadinae, which has more. In some species there is an undulating membrane. They have one or more nuclei of the vesicular type. Certain forms which feed upon solid particles have a cytostome for ingestion, while others lack such orifices and derive their nourishment by absorbing nutritive materials through the entire body surface. Some form both trophozites and cysts, while others have no cystic stage. There are a great number of species of both free-living and parasitic forms.

The Flagellates

There are five species of flagellates which can parasitize the intestines of human beings: (1) *Giardia lamblia* or *Giardia intestinalis;* (2) *Chilomastix mesnili;* (3) *Trichomonas hominis* or *Trichomonas intestinalis;* (4) *Embadomonas intestinalis;* and (5) *Enteromonas hominis.*

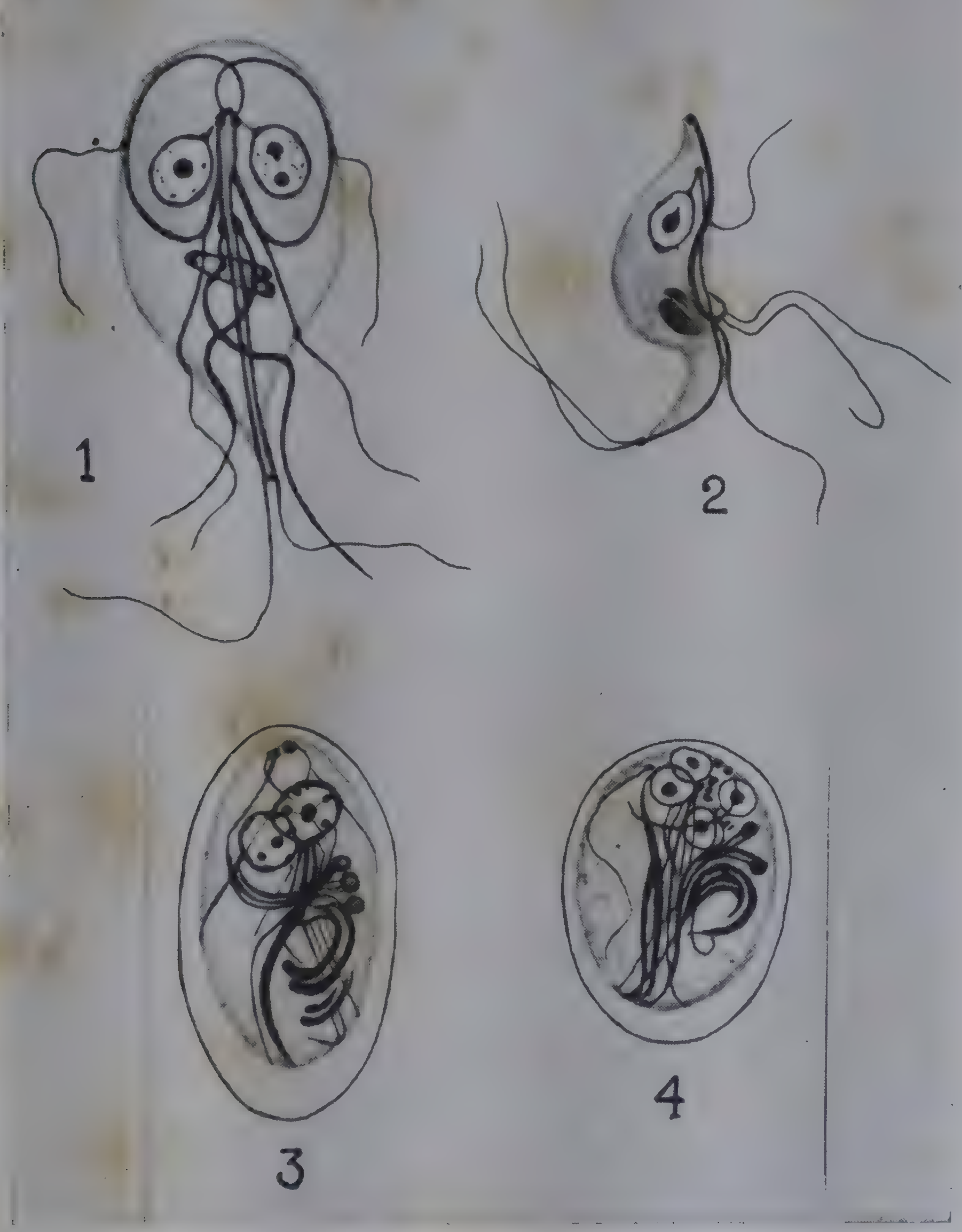

Fig. 461.—*Giardia lamblia.* *1,* Trophozoite or vegetative form, front view; *2* side view. *3,* Cystic form with two nuclei. *4,* Mature quadrinucleate cyst. (Adapted from Dobell and O'Connor by Kourí in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Family **OCTOMITIDAE**

Giardia Lamblia Stiles (1915)

The **trophozoites** or vegetative forms of *Giardia lamblia* are pyriform, bilaterally symmetrical flagellates with a broad anterior extremity and a pointed posterior end. From the suctorial disc arise six symmetrical flagella, three on each side. There is also a pair of flagella which arises from the pointed posterior end of the organism.

Cysts are ovoid with resistant covering, nonretractile, and with double contour. They have two to four paired nuclei, each with a fine central or excentric karyosome, round, and located in one of the poles. The cysts are 8 to 12 microns long and 7 to 10 microns wide.

Diagnosis is made by microscopic examination of stools when vegetative or trophozoite forms abound in the diarrheal liquid stools, and when cystic forms are found, often in large numbers, in pasty or hard stools. There are negative phases, which may last several days.

Examination of bile obtained by duodenal intubation enables one to find flagellates during these negative coprologic phases.

Staining with iron-hematoxylin is not necessary to distinguish the cysts of Giardia. Direct examination suffices, using Lugol or Gram stain.

Family **CHILOMASTIGIDAE**

The family Chilomastigidae includes flagellates with oval or pyriform bodies and four flagella, three of which are anterior and one posterior, directed backward, with the long axis parallel to the cytostome. Cysts are pyriform or lemon-shaped and are uninucleate.

Chilomastix Mesnili (Wenyon 1910) Alexeieff 1922

Chilomastix mesnili is a small flagellate with a pyriform trophozoite, 10 to 15 microns long and 5 to 7 microns wide. The broad anterior end shows three free flagella. The body is frequently twisted.

Cysts are small, 7.5 or 8.5 microns, pear-shaped, almost spherical, refractive, and colorless. They have a double-contoured wall, a nucleus, parts of their flagella, and a cytostome. As with Giardia, at times one sees gray or blue cysts of *C. mesnili* in microscopic preparations of stools stained with Lugol solution.

Some individuals are carriers of this parasite without symptoms. Others show functional changes in the digestive system.

Diagnosis.—Trophozoites are found in liquid and soft stools, while the precystic and cystic forms appear in semisolid and solid stools. Trophozoites are found primarily in recently passed stools.

It might be confusing for beginners to differentiate the trophozoites, but by using the oil immersion objective and examining only fresh preparations, this flagellate can be differentiated from *Trichomonas hominis*.

Culture.—*C. mesnili* can be cultivated in a medium composed of 1 part of inactivated human blood serum and 4 parts of Locke's solution containing a small amount of glucose.

Family **TRICHOMONADIDAE**

Trichomonadidae are flagellates characterized by a definite cystostome, a variable number of flagella, and an axostyle, a canelike structure, which arises from the blepharoplast and passes through the body to the posterior end, through which it usually protrudes. Only the human parasite, the genus Trichomonas, is of interest in human parasitology.

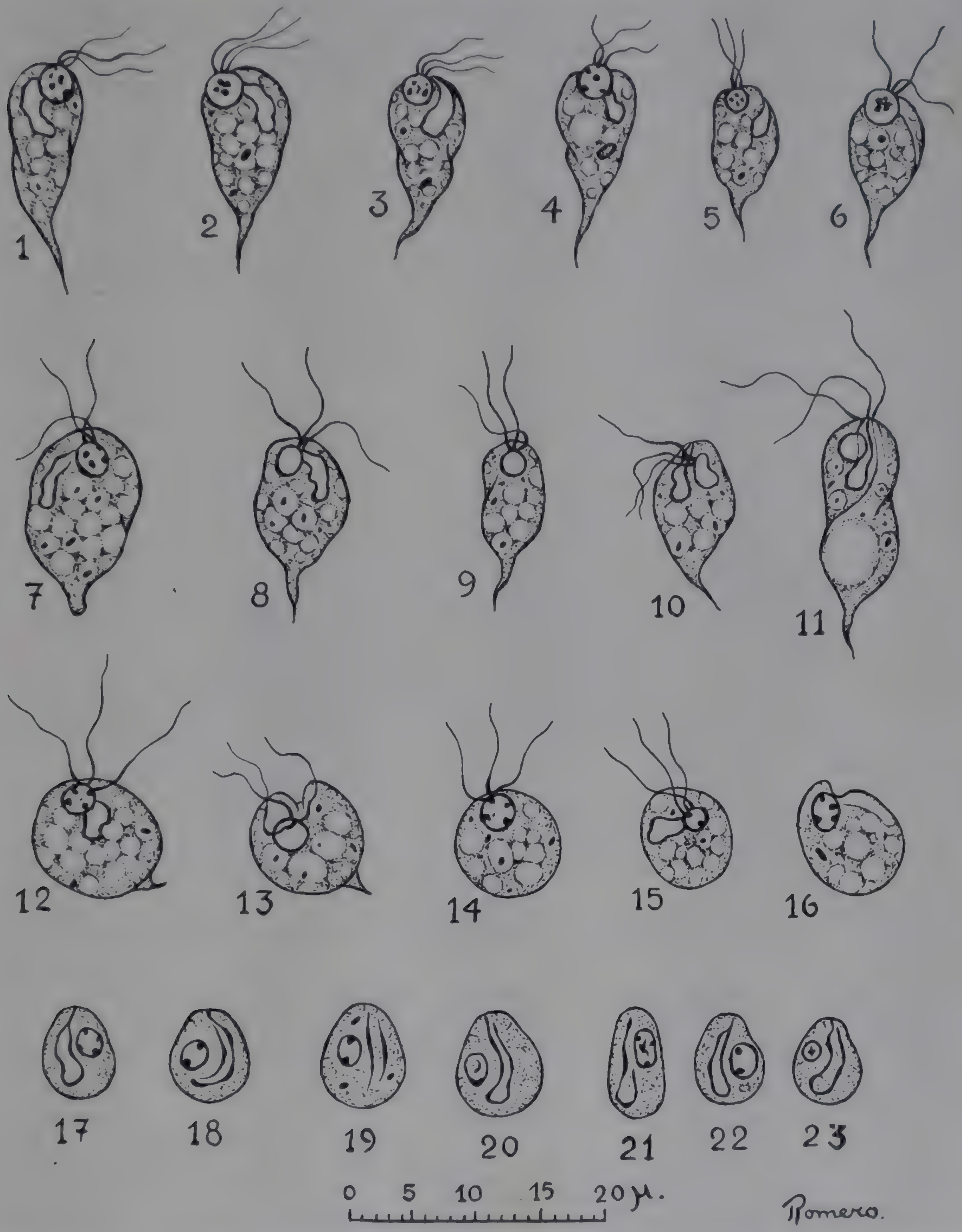

Fig. 462.—*Chilomastix mesnili.* *1* to *11*, Trophozoites. *12* to *16*, Rounded or spherical trophozoites in the process of dying. *17* to *23*, Cysts. Iron-hematoxylin stain. (After Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Trichomonas Hominis (Davaine 1860) Leuckart 1879

The *Trichomonas hominis* has no cysts. The trophozoites are almost round, 10 to 15 microns long, by 7 to 10 microns wide. They show three to five anterior flagella. In addition, there is a large and adherent posterior flagellum which forms the border of the undulating membrane as it passes down the body of the organism toward the posterior end, where it is often continued beyond the membrane.

The Trichomonades are usually considered by most authors as inoffensive parasites of the digestive canal. They are found in great numbers in the alkaline diarrheas, although they may be found as well in acid diarrheas. They can produce a chronic catarrhal disorder of the intestines.

Diagnosis is made by microscopic examination of diarrheal stools or of liquid stools after a saline purgative.

Trichomonas Vaginalis Donné 1837

Morphologically, the *T. vaginalis* and the *T. hominis* are identical except for variations which appear to be dependent upon certain conditions of the medium in which these species develop—the intestines for one and the vagina for the other. *T. vaginalis* can be seen in the abnormal secretions of the vagina, in the urine of both men and women, and also in prostatic secretions.

In **cultures,** *T. vaginalis* has the same morphology as the living form seen in the vagina. Cystic forms are also found in cultures.

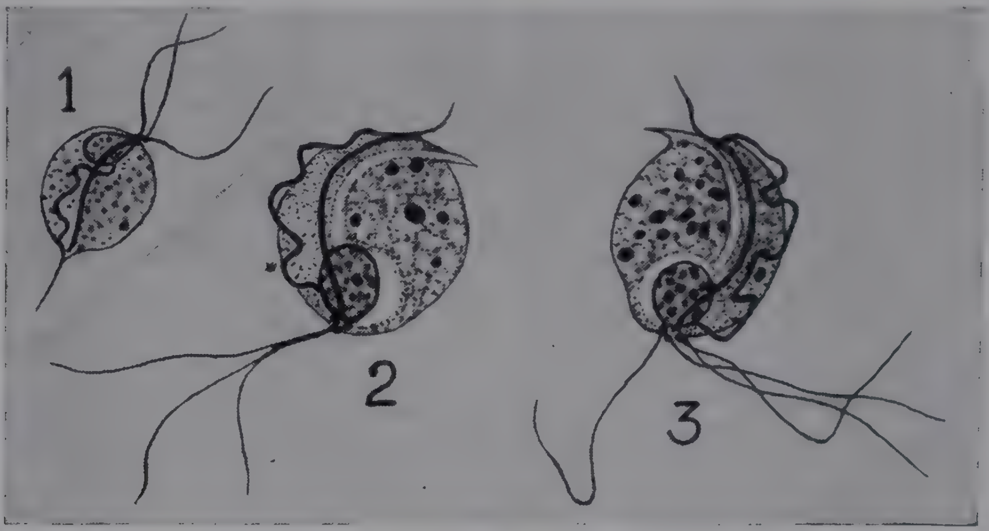

Fig. 463.—*Trichomonas hominis.* *1,* Small specimen with three anterior flagella. *2,* Large specimen with three anterior flagella. *3,* Specimen with four anterior flagella. (After Dobell and O'Connor in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

The method of **transmission** is not definitely known. It is frequently found associated with vaginitis, never in the normal vagina, so that most gynecologists consider it the causative agent of this type of vaginitis; there is much controversy on this point. It is believed that males are the carriers of *T. vaginalis* and transport this organism to women.

Diagnosis is made from direct microscopic examination, by concentration with centrifugation in salt solution, and by cultures of the vaginal secretions.

Since one of the first requirements for the establishment of a base line of efficacy of drugs and other measures in the therapy of *T. vaginalis* is a reliable criterion for cure, it is necessary to evaluate all different methods used in the diagnosis of trichomoniasis.

Kean and Day[1] have outlined the following methods for establishing this criterion of cure:

Methods.—

Patients are instructed not to douche for 24 to 48 hours prior to coming for an examination. Vaginal secretion is obtained by introducing a vaginal speculum and swabbing the mucosa with two cotton swabs. Most of the material is derived from the posterior fornix although some secretions from other portions of the vagina are also utilized.

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[1] Am. J. Obst. & Gynec. 68: 1510, 1954.

I. **Hanging Drop.**—

Introduce one swab immediately into a test tube containing 0.1 c.c. of physiologic saline at a temperature of 37° C., and mix the contents thoroughly.

Prepare hanging drops using the depressed slides in the usual manner and examine immediately. Study 3 hanging drops for a total of 10 minutes before rendering a negative report.

II. **Cultures.**—

Introduce the second cotton applicator into a test tube containing a culture medium which has been left in an incubator for 2 hours at 37° C. Rotate the swab in the medium and drop it into the test tube and leave at room temperature for 2 to 3 hours.

Place the tube in an incubator for 48 hours.

Subculture.

Examine the original cultures twice: first after 48 hours of incubation when subcultures are made, and again after 96 hours.

Make transfers by withdrawing 2 c.c. of suspension with a sterile pipette.

Use the simplified trypticase serum medium described by Kupferberg and associates[2] to which has been added penicillin and streptomycin:

Maltose	1 gm.
Bacto-agar	1 gm.
Distilled water	400 c.c.

Heat in an autoclave at 10 pounds pressure for 10 minutes to dissolve the agar, and filter.

Add	Trypticase	20 gm.
	Cysteine HCl	1.5 gm.
	0.5% Methylene blue	0.48 c.c.
	Distilled water to	600 c.c.

Adjust the reaction to pH 6.0 with 5 N sodium hydroxide. Dilute to volume (1,000 c.c.) with distilled water.

Tube in 8 c.c. amounts.

Autoclave at 15 pounds pressure for 15 minutes and cool to room temperature.

Add 0.5 c.c. sterile serum (100%) which has been adjusted to pH 6.0.

To each 8.5 c.c. tube add 2,500 units of crystalline penicillin G in 0.5 c.c. of distilled water

and 10 mg. of streptomycin in 1 c.c. of distilled water.

The final amount in each tube is 10 c.c.

No subculturing is necessary. If the seeded tube is examined after 48 hours and is negative, re-examine after 96 hours' incubation; in this way, all positives will be detected.

III. **Papanicolaou Technic.**—

Prepare vaginal and cervical smears taken routinely for the detection of cancer, and read for the presence or absence of *T. vaginalis* as follows:

Vaginal Smears:

The vaginal secretion is aspirated from the posterior fornix of the vagina by means of a glass pipette (6 inches long with a strong bulb).

Expel the secretion onto a dry glass slide and spread with the end of the pipette.

Immerse the slide immediately in fixative: equal parts of ether and 95% alcohol.

Cervical Smears:

Insert speculum into the vagina without the use of a lubricant.

Take smears with cotton applicator from the surface of the cervix, and spread this material on a slide.

Fix immediately in ether-alcohol, equal parts.

Leave the vaginal and cervical smears in the fixative for at least 1 hour, then stain by the Papanicolaou method.

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[2] Proc. Soc. Exper. Biol. & Med. 67: 304, 1948.

Kean and Day found that the Papanicolaou method is the least satisfactory for the diagnosis of *T. vaginalis.* It has the only advantages of permanence of material and opportunity for delayed reading. An inclusion of diagnosis of trichomoniasis is part of another routine, namely, cancer diagnosis, but no additional expense or effort is involved. It also correlates infection with cytologic changes produced by parasites.

Summarizing, these technics for the diagnosis of vaginal trichomoniasis indicate that a simple culture method is superior to the hanging drop and the Papanicolaou smears. The Papanicolaou method is as effective as the hanging drop, but requires too much technical skill for routine diagnosis of *T. vaginalis.* The prevalence of the parasite as determined by the culture method on a single examination was 6.5 per cent in asymptomatic ''cancer survey'' patients, 15 per cent in obstetric patients, and 26 per cent in gynecologic patients.

Family **CERCOMONADIDAE**

The family Cercomonadidae includes flagellates which have one or more anterior flagella and one posterior flagellum attached to the superficial part of the body, in a longitudinal direction.

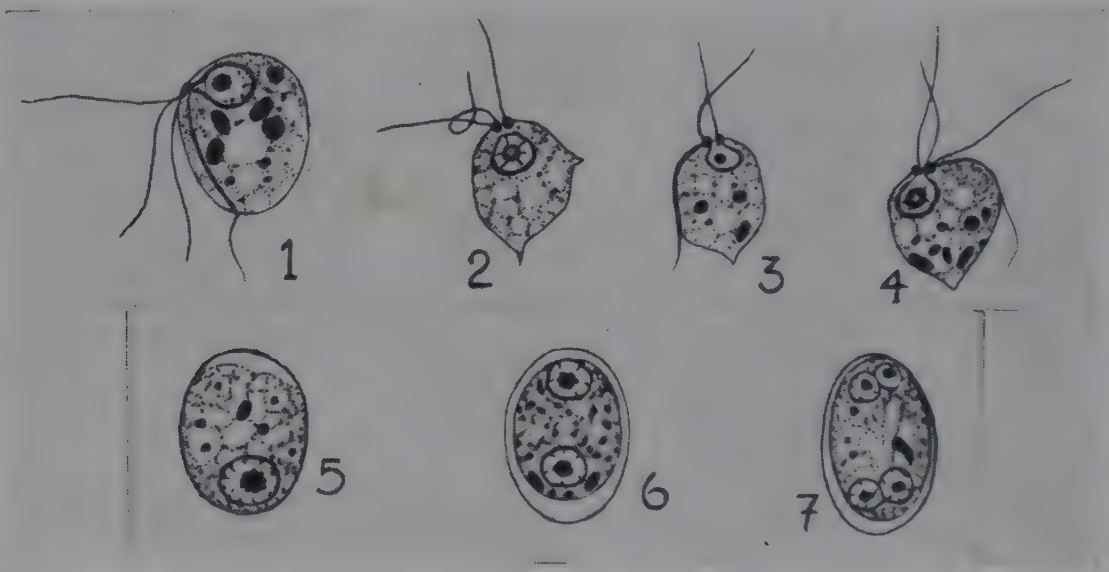


Fig. 464.—*Enteromonas hominis.* *1,* Typical form of trophozoite with four flagella, three free and one recurrent. *2,* The recurrent flagellum is not clearly visible. *3,* Only the two anterior flagella are visible. *4,* Typical form showing two blepharoplasts. *5* to *7,* Cysts uninucleate, binucleate, and quadrinucleate (mature), respectively. (In Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Enteromonas Hominis da Fonseca 1915

These flagellates are very small, pear-shaped, round, or oval, flat on one side, 4 to 10 microns long, and 3 to 6 microns wide. They have a rather voluminous anterior nucleus with dense karyosomes. There are four flagella, three of which are free; the fourth, sometimes not seen, is directed backward and adherent to the body of the parasite and terminates as a free flagellum on the posterior end of the parasite. Cysts are oval, have double-contoured walls, and are 6 to 8 microns long and half as wide. They show one or two nuclei when the cysts are immature, with four in the fully developed cysts. Two of these nuclei are in each pole. They have distinct karyosomes. Staining with iron-hematoxylin is necessary to make out the structural details.

Family EMBADOMONADIDAE

The species in the family Embadomonadidae are oval, have one nucleus anteriorly situated, a cytostome, two blepharoplasts, one anterior flagellum, and one posterior flagellum both rising from the blepharoplast in contact with the nuclear membrane. The posterior flagellum is usually contained within the cytostome before it becomes free.

Embadomonas Intestinalis Wenyon and O'Connor 1917

The *Embadomonas intestinalis* is a very small flagellate, about 4 to 9 microns long and 3 to 4 microns wide. It is usually round, although at times it is pyriform with a pointed posterior extremity. It has two anterior flagella, one of which is larger than the other, directed posteriorly and adherent to the cytostome. The other is free. In the anterior portion there is a cytostome and a rather large nucleus with a thick karyosome. The cysts are very small, 4 to 7 microns by 3 to 4 microns, pyriform.

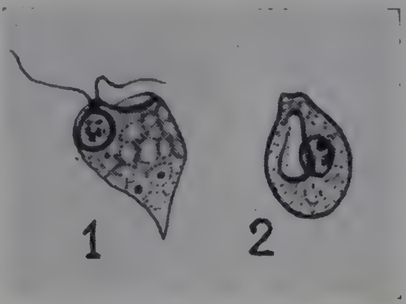


Fig. 465.—*Embadomonas intestinalis.* *1*, Trophozoite; *2*, cyst. (Adapted by Kourí from Dobell and O'Connor, in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Flagellates of the Blood and Human Tissues

Order PROTOMONADIDA

Family Trypanosomidae

In the family Trypanosomidae, genus Trypanosoma, there are three species: *T. gambiense, T. rhodesiense,* and *T. cruzi.*

In the genus Leishmania, there are three species: *L. donovani, L. tropica,* and *L. braziliensis.*

Trypanosomes cause human trypanosomiasis. Two important species, *T. gambiense* and *T. rhodesiense,* are specific agents of African sleeping sickness or trypanosomiasis africana. The first is found in West Africa, the second in East Africa.

T. cruzi is the agent of trypanosomiasis americana or Chagas' disease.

Species of the genus Leishmania are the cause of human leishmaniasis, which occurs in three forms. (1) Visceral leishmaniasis affects the spleen; it is called "kala azar." It is endemic in tropical Asia. Infantile kala azar, or infantile splenic leishmaniasis, is found along the Mediterranean littoral. Both types are caused by *L. donovani.* (2) The cutaneous forms of leishmaniasis are found in the Near East, are known there as "oriental boil," and are caused by *L. tropica;* in America (3), it is known as "leishmaniasis americana." This is characterized by mucocutaneous ulcerations affecting especially the mouth, the nose, and pharynx.

Trypanosomes

Trypanosomes are hemoflagellates. They show a nucleus and a blepharoplast, from which proceeds a flagellum with an undulating membrane. The flagellum projects out from the forward end.

These trypanosomes are found in the blood stream of infected animals. They are transmitted through the intermediation of other animals, such as blood-sucking worms, insects, and flies. There are certain forms found at times among trypanosomes, known as Crithidia, where the blepharoplast lies ahead of, instead of behind, the nucleus. It is an insect form found in the tsetse fly, and a normal inhabitant or parasite of the mosquito.

Division of trypanosomes in mammalian animals takes place in the blood stream. This is characterized by division of the nucleus. The cytoplasmic body swells and the blepharoplast divides.

In certain types of trypanosome infections, it is very difficult to find these parasites in the blood. Especially is this the case with the nonpathogenic beef trypanosomes called the *T. theileri* and the sheep trypanosome called the *T. melophagium*. The latter form can be seen in the circulation of the so-called "sheep louse," which is found on most sheep. It is a curious fact, however, that while these sheep lice will apparently communicate this *T. melophagium* to sheep, it is very difficult to find them in their blood. Various organs of the body will show trypanosomes, the lymph vessel system, the spleen, liver, kidneys, bone marrow, and brain. The brain particularly is an object of investigation in seeking for the trypanosomes of African sleeping sickness of man. The skin and mucosa show changes.

Cultivation Methods

Successful cultivation of *T. gambiense* and *T. rhodesiense* is possible but often is a failure. It is not necessary to utilize it unless blood spread and juice examinations fail.

It is in Chagas' disease that cultivation methods offer most brilliant promise of success.

It is likewise true that animal inoculation affords a better chance of success in Chagas' disease than in East or West African sleeping sickness. Even though no *T. cruzi* are found in the blood examination, inoculation of guinea pigs will often bring success in proving the existence of Chagas' disease. The method is to inoculate from 0.5 to 1.0 c.c. of blood from the patient intraperitoneally into several pigs.

The various methods advocated by different workers for cultivating trypanosomes are as follows:

Artificial Culture Methods

Cultivation of certain trypanosomes upon artificial culture media is very successful. We have not been successful in cultivating the trypanosome of African sleeping sickness. It must be noted that, in cultures, a great many of the Crithidia forms are seen. The best temperature for cultivation is a high room temperature. The ordinary incubator temperature of 37° C. is too high for their growth.

The culture media are as follows:

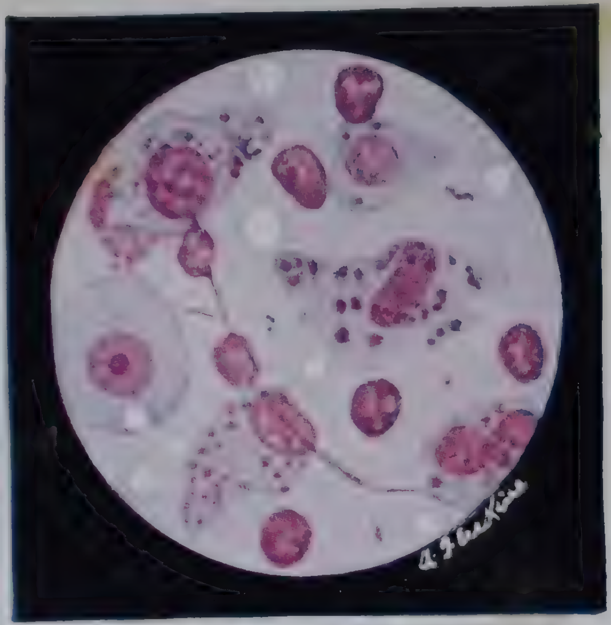
1. **Blood Broth** was originated by Miyajima in 1907. To 10 parts of ordinary weak alkaline, weak acid, or neutral broth, add 2 to 10 parts of defibrinated blood. This blood can come from the infected animal or from a noninfected animal.

2. **Blood-Corpuscle-Free Bouillon**, by Noller's method, 1913. Pipette off the clear part of the above-noted blood bouillon and use this.

3. **Fluid Medium of Row**, 1912—one part of sterile, defibrinated rabbit blood to 8 or 10 parts of sterile water. After hemolysis has taken place, to every 100 c.c., add 0.8 gram of sterilized sodium chloride.

KALA AZAR

SPLEEN SMEAR



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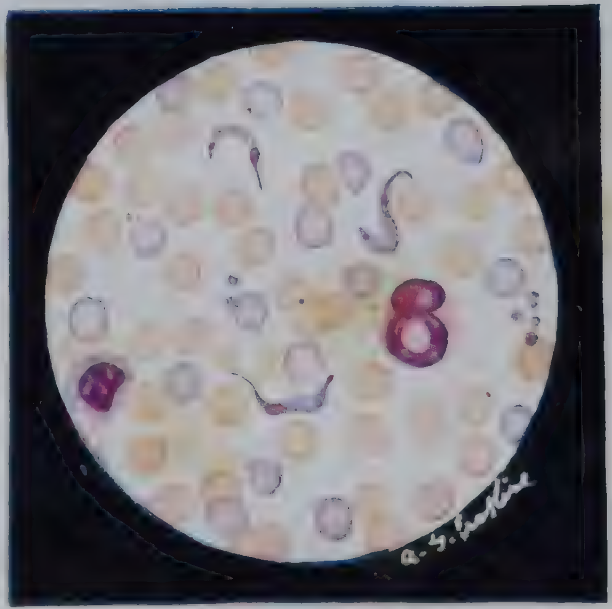
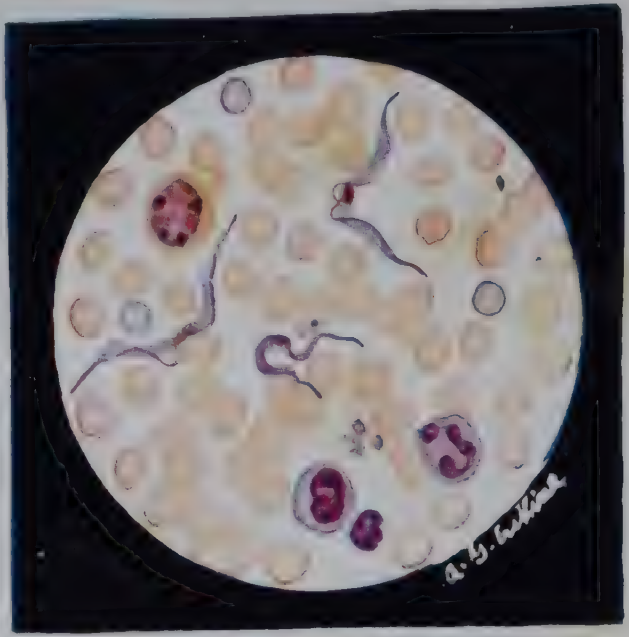


CHAGAS' DISEASE

TRYPANOSOMA CRUZI

SURRA OR MAL DE CADERAS

TRYPANOSOMA EVANSI



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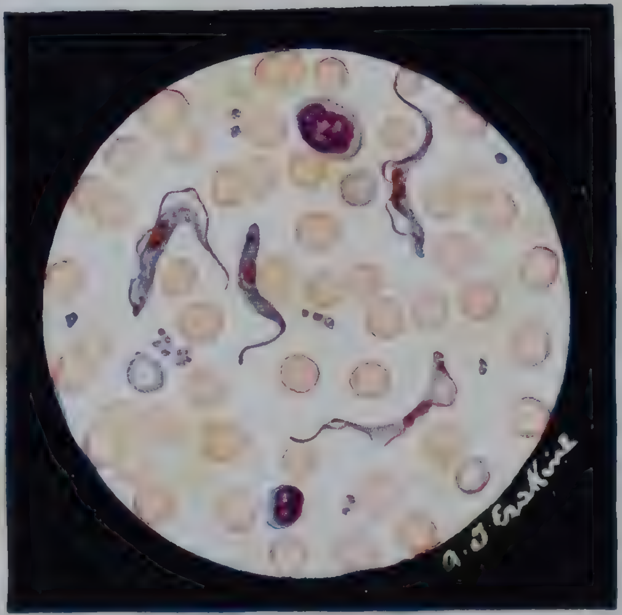
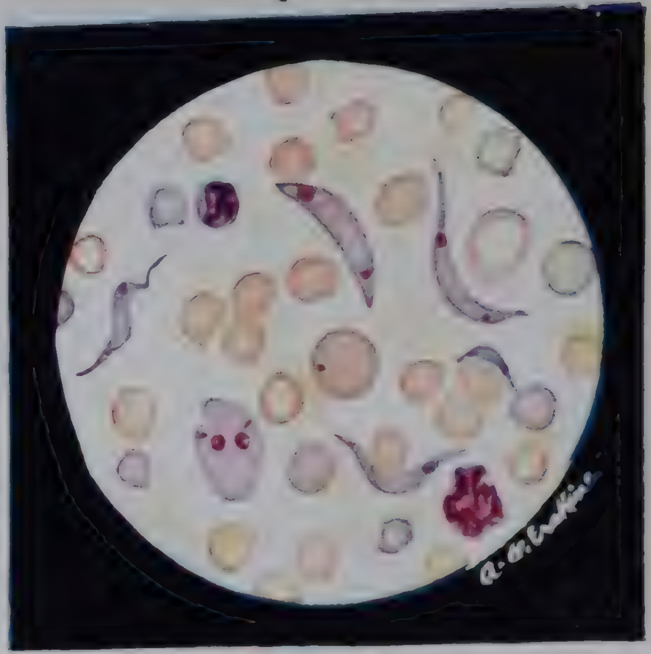


NAGANA

TRYPANOSOMA BRUCEI

(RAT)



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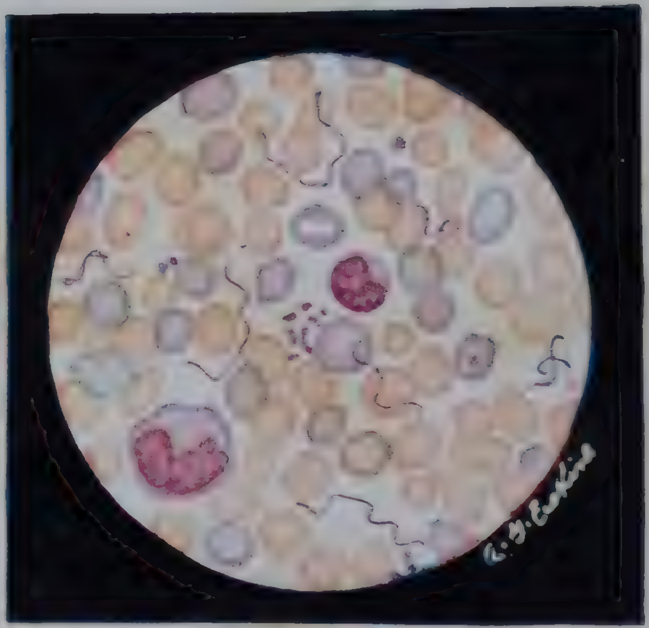

EUROPEAN RELAPSING FEVER

BORRELIA RECURRENTIS

PLATE XLVII.

4. **Hypotonic Rabbit Blood,** by Ponsel, 1923. One part of sterile fluid defibrinated rabbit blood is mixed with one part redistilled water. Place in test tubes and inactivate for half hour at 60° C.

5. **Medium of Wenyon and Noguchi,** 1921: To 270 c.c. of 0.85% physiologic salt solution (reaction pH 7.6) add 30 c.c. of ordinary 2% nutrient agar. After mixing, place 10 c.c. in test tube. Autoclave at 120° C. and after cooling to 50° C. add 20 drops of fresh rabbit blood from the disinfected ear of the rabbit to each test tube. Mix without shaking and incubate at 37° C. for 24 hours to determine sterility. Then use the tubes.

6. A solid culture medium which is much used is the blood agar medium of Novy and McNeal, 1903. This is the so-called **N. N. Agar.** Infuse 125 grams of chopped-up beef in 1,000 c.c. of distilled water, strain, add agar 20 grams, peptone 20 grams, sodium chloride 5 grams, normal sodium carbonate solution, 10 c.c. The agar is placed in tubes and mixed with blood at 55° C. The blood volume should be about one-fifth of the agar volume.

7. **Grape Sugar Blood Agar Medium of Hagestermeister:** Hagestermeister used the N. N. Medium with 0.5% sodium chloride and 1% peptone, weakly alkaline, with 2% grape sugar added to this. He used defibrinated horse blood.

8. **Blood Agar Medium of Novy, McNeal, and Nicolle (N. N. N. Medium).** This is especially designed for Leishmania cultivation but it is also good for trypanosomes. Nicolle, in 1908, described it as follows: Agar 14 grams, salt 6 grams, distilled water 900 c.c.

It is made just like N. N. Agar. Add rabbit blood when the agar has cooled to 52° C., usually one-fifth as much blood as agar.

9. **Kelser's Medium.** This medium has been described by Kelser and is a modification of that previously described by Bonacci. Prepare it as follows:

Bacto-peptone	12.5 gm.
Sodium chloride	3.5 gm.
Agar (Bacto-agar, granular)	5.0 gm.
Bacto-beef, dehydrated	25.0 gm.
Distilled water	500.0 c.c.

Add the beef to the 500 c.c. of water and place over a water bath at 55° C. for one hour. Now add the peptone and the sodium chloride. Bring the mixture to the boiling point and hold it there for five minutes. Shake and filter through cotton. Neutralize to litmus by using N/1 sodium hydroxide.

Make it 1 per cent agar. After the agar has dissolved, place in test tubes or Erlenmeyer flasks, sterilize at 12 pounds in the autoclave, and it is ready for use. Use by melting in water bath, keeping the temperature at 50° C. Add to each tube 0.25 c.c. of a sterile 1 per cent dextrose solution and 0.25 c.c. of fresh, sterile, defibrinated guinea pig blood.

Slant the tubes so as to have a deep butt and a short slant. If such media are kept on hand and become dried out, add a little of the dextrose broth solution when ready for use. This dextrose broth solution to overcome loss of water of condensation is made by adding one part of sterile 1 per cent dextrose solution to ten parts of peptone broth prepared as described above. According to Kelser's technic, maintain a stock culture of *T. cruzi* by covering cotton plugs with rubber cap to prevent evaporation.

Inoculate medium by means of sterile glass capillary pipettes closed with cotton air filters, equipped with rubber nipples. Transfer cultures every ten days.

Trypanosoma Gambiense—Trypanosoma Rhodesiense African Sleeping Sickness*

Sleeping sickness or African trypanosomiasis is often designated as "African sleeping sickness," to distinguish it from Economo's lethargic encephalitis which also formerly was called "sleeping sickness." It is a disease caused by trypanosomes, and is characterized by chronic fever, more or less intensive glandular enlargement, and, in many cases, a cerebral stage with fatal outcome.

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*After O. Felsenfeld in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine. 1948. The C. V. Mosby Co.

Geographical Distribution.—The geography of sleeping sickness follows the African belt of the tsetse flies (Glossina), mainly of *Glossina palpalis, G. tachinoides, G. morsitans,* and *G. swynnertoni,* the most frequent insect vectors of the disease. The northern boundary of sleeping sickness approximately follows a line drawn from St. Louis to the Lake Chad, then along the Shari and the southern part of Anglo-Egyptian Sudan. Abyssinia and Italian Somaliland are free from it. Southwest Africa and the Zambezi River, with the exception of two foci, are the southern boundaries, while in West Africa, along the Gulf of Guinea, in the Congo, in Angola, Sudan, Uganda, and Kenya, the classic type ascribed to *Trypanosoma gambiense* prevails, and the Rhodesian type ascribed to *T. rhodesiense* is found in Tanganyika, Mozambique, Nyasaland, and Rhodesia.

Etiology.—Sleeping sickness is caused by trypanosomes. These organisms are similar to *Trypanosoma brucei,* the agent of the animal disease "nagana." Two types of trypanosomes have been described as the cause of sleeping sickness: *T. gambiense* and *T. rhodesiense.* It is not possible to differentiate between these types morphologically or by animal experiments, except by production of keratitis in goats by *T. rhodesiense.* It was formerly believed that the forms of *T. rhodesiense* in the blood of injected mice or rats more frequently have their nuclei located more posteriorly than do forms of *T. gambiense* under similar experimental conditions. Some authors consider *T. rhodesiense* a human-adapted variant of *T. brucei.* Others ascribe the difference in clinical types of sleeping sickness to the adaptation of the trypanosomes to various species of Glossina.

The organisms appear irregularly in the blood but are usually present during the periods of fever. They are most easily found in enlarged lymph glands, later occurring in body fluids, principally the cerebrospinal fluid. Aside from elongated, slender trypanosomes, rather short, broad forms with smaller flagella and posterior nuclei may be observed. After the organisms enter the tsetse fly by feeding on a patient who harbors trypanosomes, they multiply in the midgut, later in the hindgut, of the Glossina. After 12 to 36 days, depending on the temperature, humidity, and other factors, the infective metacyclic forms reach the salivary glands of the fly and may be injected into a susceptible host during a blood meal.

Trypanosomes of sleeping sickness are difficult to cultivate. They are, however, easily transferred to laboratory animals, chiefly to mice, rats, and guinea pigs.

Vector and Animal Reservoirs.—The trypanosomes are usually transferred by tsetse flies after they have gone through a cyclic evolution in these insects. There is, however, a possibility of purely mechanical transfer by Glossinae and other biting insects from person to person under certain circumstances.

The principal species of Glossina which transfer sleeping sickness are *G. palpalis* and *G. tachinoides* for *T. gambiense,* and *G. morsitans* and *G. swynnertoni* for *T. rhodesiense.*

Most species of Glossina require shade and proximity of water. Glossinae which live in southwest Arabia are not infected with trypanosomes. The organisms are not transferred to the larvae of the flies. No repellent effective against tsetse flies is known.

Resistance.—There is no racial predisposition to sleeping sickness. Neither does the disease prefer people of one sex or of a certain age group. Trypanosomes are not, as a rule, transferred from mother to fetus, but some exceptions have been described.

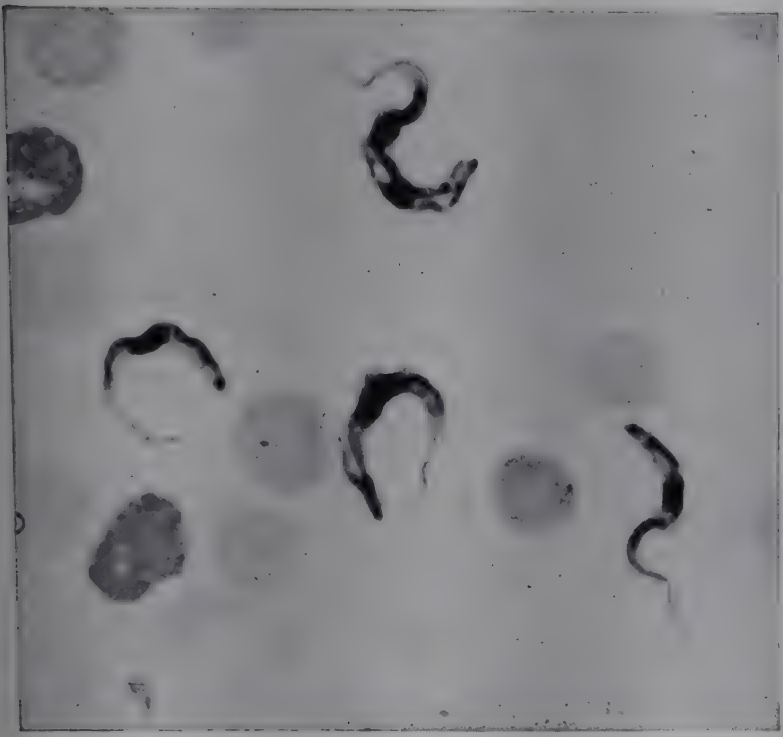

Fig. 466.

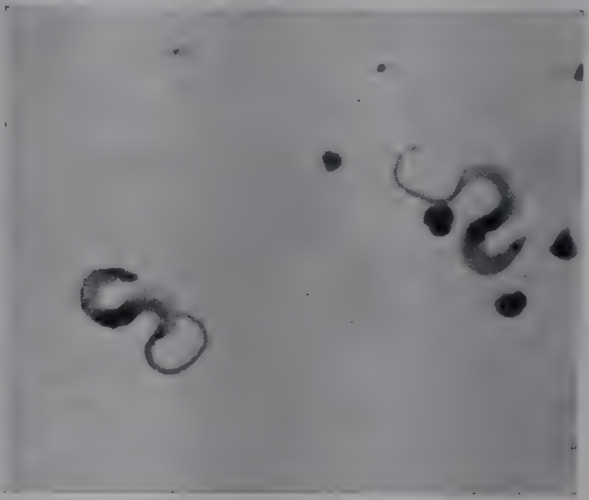

Fig. 467.

Fig. 466.—*Trypanosoma gambiense* in peripheral blood. (O. Felsenfeld, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Fig. 467.—*Trypanosoma rhodesiense* in peripheral blood. (O. Felsenfeld, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Laboratory Diagnosis.—The number of parasites in the peripheral blood undergoes considerable fluctuation. The parasites are not easily recovered from the cerebrospinal fluid in every case. Gland puncture is the method of choice for recovering the parasites if there is enlargement of the lymphatic glands.

(A) THE PERIPHERAL BLOOD

Cultivation of the trypanosomes is not as yet practical for diagnostic purposes.

For animal inoculation, guinea pigs are used most often. Inject 5 or 10 c.c. of blood intraperitoneally.

For examination of the blood for trypanosomes, use the thick drop method (pages 650 ff.), or centrifuge citrated blood and examine the sediment.

Both stained and unstained preparations are examined. The Giemsa method and its modification by Field are the recommended methods of staining.

(B) THE LYMPH GLANDS

Puncture the lymph glands after massage, using a relatively strong needle and a 10 or 20 c.c. syringe.

Prepare spreads and stain by the Giemsa or Field method.

Strict asepsis is necessary for this operation, but no anesthesia is needed. The lateral cervical glands are easily accessible, particularly when they are enlarged. Massage of the glands during puncture enhances the operation.

Examine both stained and unstained spreads.

(C) CEREBROSPINAL FLUID

Centrifuge the spinal fluid and observe the sediment microscopically.

The sediment can be used for injection into guinea pigs, or for cultivation.

Trypanosomes are often recovered from the fluid gathered through suboccipital puncture and also from sternal puncture material.

(D) SEROLOGIC REACTIONS

The so-called ''adhesion reaction'' is sometimes used for diagnostic purposes. This consists of the adhesion of human red blood cells to trypanosomes, when trypanosomes are mixed with immune serum, complement, and red blood cells. In the presence of a nonspecific serum, no such clumping or adhesions occur. Serologic reactions, however, have not as yet attained an important place in the diagnosis of sleeping sickness.

(E) MISCELLANEOUS

The **blood picture** shows secondary anemia. The number of white blood cells is within normal limits in the beginning of the disease. Leukocytosis appears during the later stages of sleeping sickness. There is a relative increase of lymphocytes and monocytes.

Autoagglutinins occur often, causing rouleaux formation of the red blood cells. Cold agglutinins have not been found.

Blood Chemistry.—The blood sugar and the alkali reserve of the blood decrease during the disease. The serum is depleted of proteins, chiefly of the albumin fraction. The albumin-globulin ratio is low. Nonprotein nitrogen, urea, and creatinine increase in the terminal stage, while the cholesterol usually decreases.

Spinal Fluid.—During the cerebral stage, the spinal fluid contains many cells, mostly lymphocytes and plasmacytes. The occurrence of plasmacytes in the cerebrospinal fluid is of diagnostic value.

A considerable amount of globulin is usually present in the spinal fluid. Pandy, Nonne-Apelt, and Weichbrodt reactions are positive. The colloidal gold curve shows the paretic type.

Trypanosoma Cruzi
Chagas' Disease or Trypanosomiasis Cruzi

The term ''trypanosomiasis americana'' is incorrect in describing this disease, since the parasite has been found in primates in Java and the vector has a wide geographical distribution.

Trypanosoma cruzi is about 15 microns long, with the blepharoplast situated near the outer point. Dividing forms are not found in the blood stream, but are found in the tissues, without flagella, in this way simulating the form of the organism of kala azar, but are larger than the Leishmaniae of kala azar.

T. cruzi can easily be grown in blood agar. It is pathogenic for monkeys, cats, guinea pigs, dogs, and mice.

Infection is carried through a large bug, the *Triatoma megista*. This triatome is 3 to 3.5 cm. by 1 cm. Light red bands are seen over the abdominal segments. The thorax shows similar stripes or bands. All stages in the development of this bug suck blood.

Chagas' disease is an acute disease with anemia and nervous, cardiac, and myxedematous symptoms.

Diagnosis.—Diagnosis is made by direct or indirect examination.

Direct Examination

Fresh Specimen.—The fresh specimen is very seldom used. It can be useful, however, in very acute cases with heavy parasitism. The parasites should be searched for with high magnification. The serpentine movements of the parasite can be observed as it rapidly traverses the microscopic field.

Thin Blood Film or Thick Drop.—The thin film is not recommended except in acute cases or in heavy parasitism of animals where intact forms of the trypanosomes can be attained. The thick drop is indicated because it increases the probability of finding the parasites. Ross states that in a thin drop of blood only one parasite might be found when in reality there may be as many as 150,000,000 parasites present.

Thick Drop of Blood (Ross) (Errecart's Modification).—This is the best method of examining stained specimens of blood and is very useful for epidemiologic studies. The parasites are fragile and are easily deformed during the examination. In order to prevent breaking the parasite, the following solution should be used.

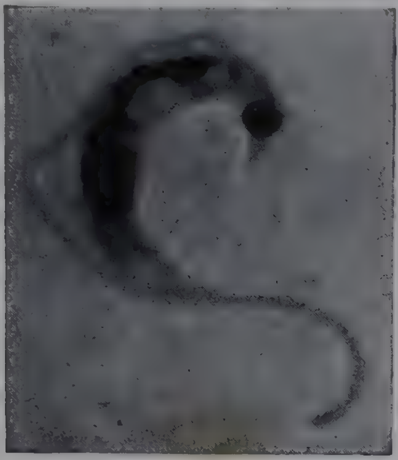

Fig. 468.—*Trypanosoma cruzi* in human blood. Giemsa stain. (×2,000.) (Mazza, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Errecart Solution

Formaldehyde, 40%	1 c.c.
Acetic acid	0.2 c.c.
Physiologic saline	100 c.c.

Gently mix, with a loop, 3 or 4 drops of blood with 1 drop of the solution. Make a very thin spread from this.

Dry in the air, but take care to protect it from flies, dirt, etc.

Dehemoglobinize with distilled water. If dehemoglobinization is not complete, use a solution of 0.2 to 0.5 per cent acetic acid. If the 0.5 per cent solution is used, wash with distilled water several times.

Stain with Giemsa stain diluted 1 drop to each c.c. of neutral distilled water, for 20 minutes.

Renew the Giemsa stain and continue staining for 70 minutes more.

Wash and dry.

Cultures.—Many culture media have been proposed for *Trypanosoma cruzi:*

Geiman Medium, Modified by Groot and Hernández (used also in the cultivation of *Bartonella bacilliformis*).—

Proteose-peptone No. 3.	20 gm.
Bacto agar	20 gm.
Sodium chloride	5 gm.
Distilled water	1000 c.c.

Dissolve and adjust the reaction to pH 7.6 to 7.8 and sterilize in the autoclave. To 75 c.c. of this medium, liquefied and cooled to 45° C., add 25 c.c. of fresh rabbit blood and 0.2 c.c. of the following mixture sterilized by filtration:

Ascorbic acid	10 mg.
Glutathione	40 mg.
Ringer's solution	20 c.c.

The modification consists of adding water of condensation, 2 to 5 c.c., to the tubes, according to the size of the tubes. This is the solution which is usually added to the Kesler medium:

Bacto-peptone, Difco	12.5 gm.
Sodium chloride	2.5 gm.
Bacto-beef, dehydrated	25.0 gm.
Glucose	2.5 gm.
Distilled water	750 c.c.

Inoculate each tube with 0.5 c.c. to 1.5 c.c. of blood and incubate at 22 to 30° C. Some advise defibrinating the blood or placing it in an anticoagulant like sodium citrate. The material may be concentrated by centrifuging. Hernández and Groot found leishmanial forms after nine days, and two or three days later the Trypanosoma appeared, passing through the intermediate leptomonas and crithidia forms. They advise replanting every ten days.

Direct Method of Examination of Living Tissues.—

Biopsy of Glands (Method of Mazza-Talice).—

Select a neighboring gland which gives a 90% positive Leishmania finding, or any lymphatic gland which gives 30% positive findings.

Infiltrate a local anesthetic, 1% Novocain.

Cut the gland in half and rub against a slide.

Fix, and stain with one of the Romanowsky stains; or fix the section in 10 to 15% Formalin, and stain cut sections with iron-hematoxylin-eosin.

Cutaneous and conjunctival biopsy is easily carried out.

Cardiac and hepatic viscerotomy is a method recommended by Mazza for the diagnosis of Chagas' disease. It is similar to that used in the diagnosis of yellow fever. A small block of tissue, 1 cm. by 1 cm., may be obtained by means of an apparatus called "viscerotome," so that the cadaver need not be opened too much. In Venezuela, several chronic cases of this disease have been diagnosed by this procedure.

Mazza proposed that this method be used as a systematic procedure in all endemic zones, or in regions which show a high infestation rate with the winged bedbug.

Indirect Examination

The **complement fixation test** in the diagnosis of Chagas' disease is quite reliable. It has been especially recommended by Kelser, of the United States Army, who used the antigen prepared by making an extract of cultures grown on Kelser's medium.

Make cultures of *T. cruzi* on Kelser's medium and incubate.
Add 0.75 c.c. of sterile distilled water to each tube.
Mix with the surface growth by means of a sterile glass pipette.
Collect the mixtures from these tubes into a 15 c.c. conical centrifuge tube.
Centrifuge for one-half hour at 4,000 r.p.m.
Pipette off the supernatant fluid.
Add 8 c.c. of sterile physiologic saline.
Repeat the centrifugalization to wash the trypanosomes. Pipette off the saline.
Add 2 volumes of 50% glycerin in physiologic saline.
Mix the trypanosomes gently with a platinum loop.
This is the stock antigen and must be kept in a refrigerator.

Leishmania and Leishmaniasis

Leishmanias are small protozoa belonging to the family Trypanosomidae. They are round, oval, or cigar-shaped, 1.5 microns at the shortest diameter and 5 microns at the longest. Besides a relatively large nucleus excentrically placed, there is a parabasal body at an angle to it, and a kinetoplast. Near this is the rhizoplast which represents the axoneme of the flagellate form. With Giemsa or Wright stain, the cytoplasm is a faint blue; all other structures are red or purple.

Leishmanias are found in nature in the blood and reticuloendothelial cells of various mammals and also in sandflies in which the flagellated leptomonas forms develop. They can be cultivated in citrated blood, hemoglobin liver broth, and in the condensation water above N.N.N. medium. In cultures, they elongate, and the large flagellum grows from the kinetoplast at the blunt anterior end. Multiplication of these leptomonas continues by longitudinal fission, whereas the intracellular leishmanias in the mammalian host reproduce by binary fission.

There are three principal species:

Leishmania braziliensis causes mucocutaneous leishmaniasis, mostly in South America, and espundia, or uta.

Leishmania tropica causes cutaneous leishmaniasis and oriental sore.

Leishmania donovani causes visceral leishmaniasis, kala azar, and infantile kala azar.

Animal reservoirs are dogs, cats, and possibly the hamster. Laboratory animals in order of susceptibility are hamsters, monkeys, mice and rats, guinea pigs, and rabbits which are often refractory.

Leishmania Braziliensis

Mucocutaneous Leishmaniasis* (Espundia or Uta)

The etiological agent of mucocutaneous leishmaniasis is the *Leishmania braziliensis*. The nonflagellate or leishmania form is found in the human tissues and in animals susceptible to inoculation with the parasite; the flagellae or leptomonas form is found in the digestive tube of the vector host and in cultures. At times the nonflagellate form is found in the invertebrate host and the flagellate type might be seen, very rarely, in the vertebrate host.

Forms in the Vertebrate Host.—The leishmania forms appear often within the cells of the reticuloendothelial system; in smears they generally appear outside the cells, due to rupture of the cells while preparing the slides. The typical leishmanias are oval, with one extremity more rounded than the other. They measure from 2 to 4 microns by 1.5 to 2.5 microns. This form is susceptible to variations. The distinguishing features of the parasite are the cytoplasm, the nucleus, and the kinetoplast.

Forms in the Insect Vector.—In the insect vector, the leishmania forms rapidly evolve to produce the flagellate form.

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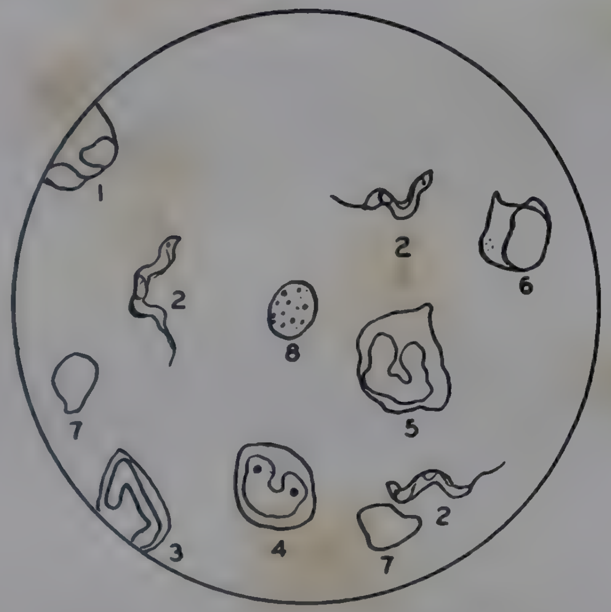
*After Pessôa in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine, 1948, The C. V. Mosby Co.

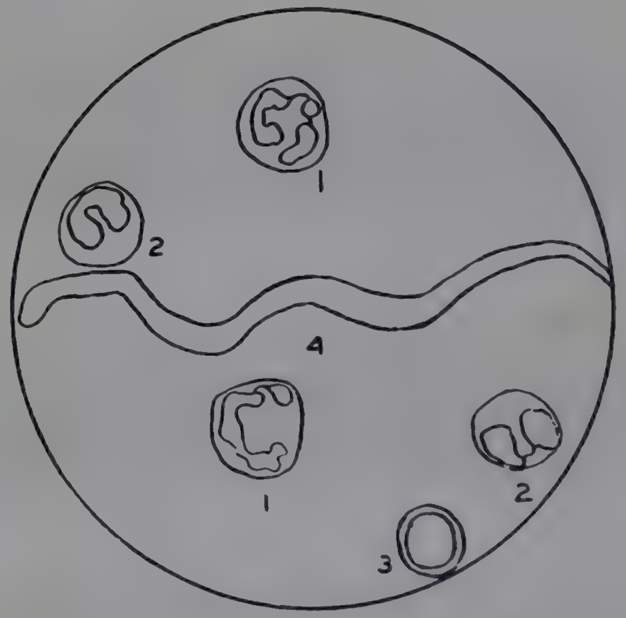
BLOOD FILMS
GIEMSA STAIN ×950



TRYPANOSOMA GAMBIENSE

1. Eosinophile
2. *Trypanosoma gambiense*
3. ''Stab''
4. Neutrophilic juvenile
5. Monocyte
6. Lymphocyte
7. Polychromatic erythrocyte
8. Basophilic punctation, coarse

Note rouleaux formation of the erythrocytes



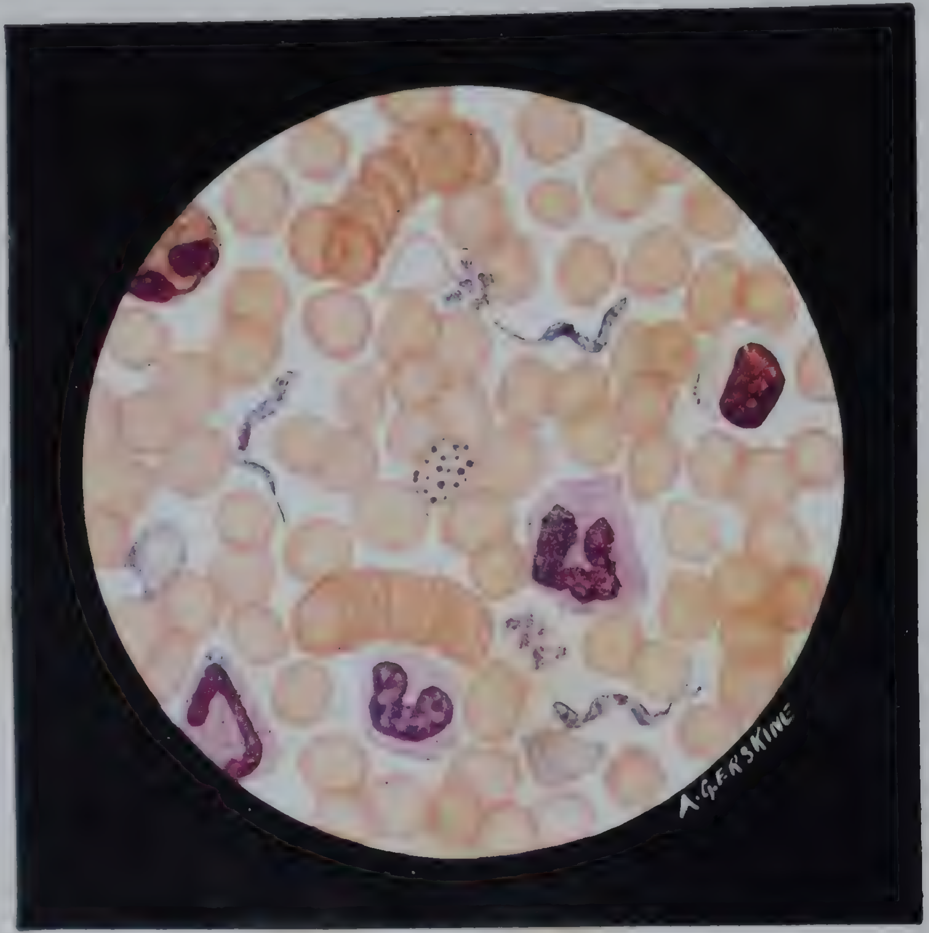
WUCHERERIA BANCROFTI

1. Eospinophile
2. Eosinophile
3. Lymphocyte
4. *Wuchereria bancrofti*

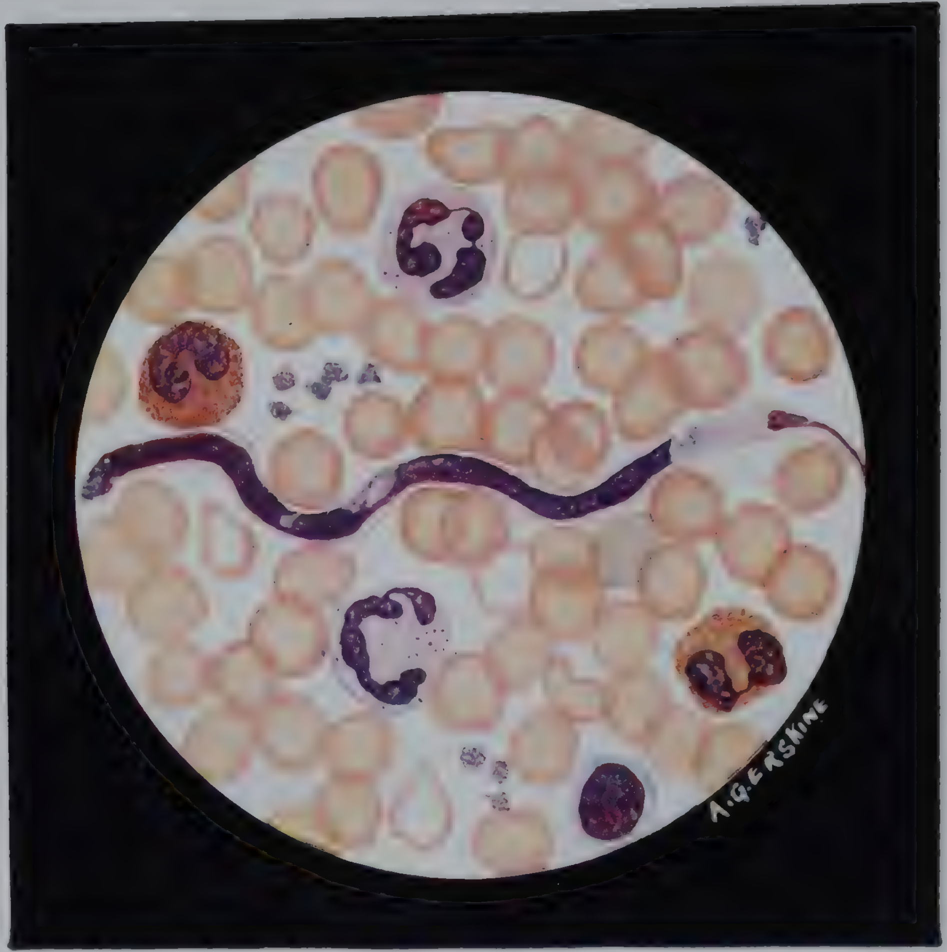
PLATE XLVIII

BLOOD FILMS

GIEMSA STAIN X 950



TRYPANOSOMA GAMBIENSE



WUCHERERIA BANCROFTI

PLATE XLVIII.

Geographical Distribution.—Mucocutaneous leishmaniasis has been found throughout most of Latin America, from Mexico to the northern part of Argentina, with a high incidence of the disease in parts of Peru and Brazil. In Brazil, the disease is most frequent in São Paulo.

Culture.—*L. braziliensis* can be cultivated in N.N.N. medium. The organisms grow in the water of condensation.

Transmission.—The intimate relationship between the incidence of Phlebotomus and mucocutaneous leishmaniasis has been noted for many years. The species which seem to play a predominant role in the dissemination of the disease are *Phlebotomus whitmani, P. pessoai,* and *P. migonei.* The *P. fischeri* seems to be the transmitter in regions of sporadic cases.

After feeding on individuals or animals infected with *L. braziliensis,* the Phlebotomus becomes infected. About 0.22 per cent seem to be naturally infected.

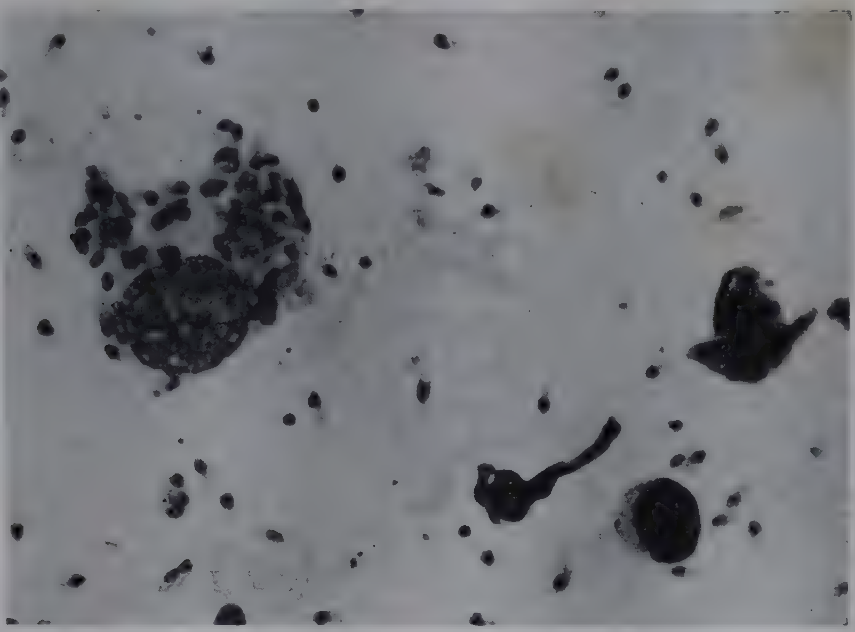


Fig. 469.—Smear of material obtained from puncture of a nonulcerative lesion of mucocutaneous leishmaniasis; a macrophage containing 62 leishmanias may be observed. (Giemsa stain.) (After Pessôa and Barretto in I. Meeting of Derm. Syphil., Brazil, 1944.) (From Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

The possibility of transmission by direct contact has been accepted by several authors.

Nonhematophagous arthropods have been incriminated as the transmitters of the disease, but this idea is not accepted. American leishmaniasis is a disease acquired in the forests, where flies are not as abundant as in cities. The disease is endemic only in certain definite regions, whereas the housefly and other flies can be found everywhere.

The Phlebotomus is probably the only hematophagous arthropod which plays an important role in the transmission of American leishmaniasis.

Laboratory Diagnosis.—The most important laboratory test is the demonstration of the parasite by direct methods: finding it in the ulcers, glands, or by culture. Curettage, puncture, or biopsy may be used to demonstrate the parasites in material taken from lesions of the skin and mucosa as well as from glands.

Place the material immediately in culture media (N.N.N. agar), or fix and section for microscopic examination. In closed lesions, puncture with a large needle is advisable.

To find the parasites in the **nasal mucosa,** curettage must be performed after anesthesia with Neotutocaine. The material obtained must be spread on slides and fixed and stained by the Giemsa method.

Cultures increase the possibility of finding the parasite. It must be kept in mind that leishmanias do not grow in the presence of other microorganisms, so that several tubes must be used to make the culture.

Complement fixation tests are complicated and not satisfactory because of lack of a good antigen.

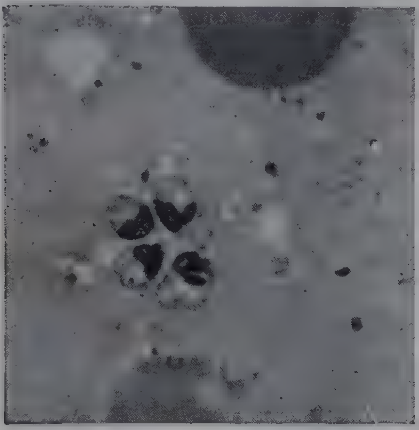


Fig. 470.

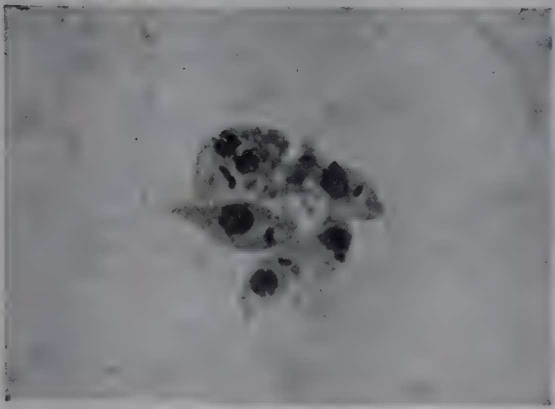


Fig. 471.

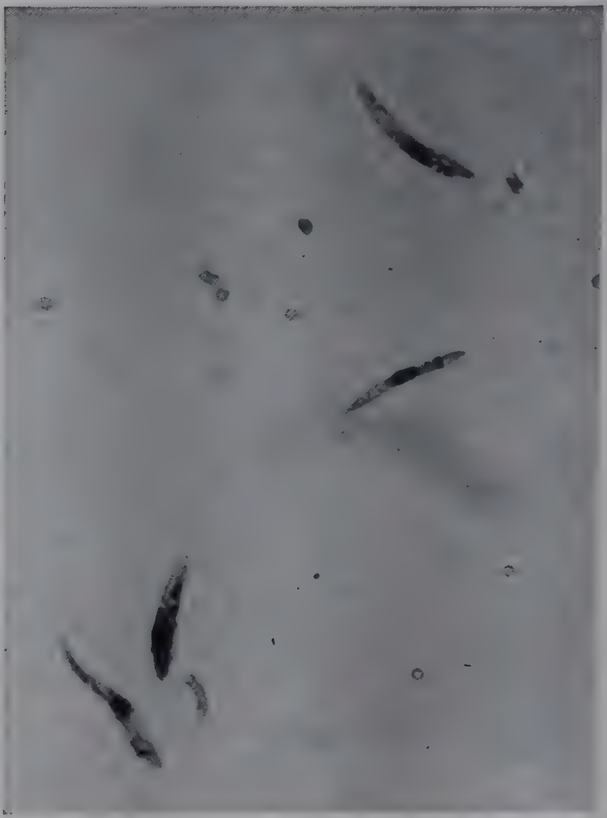


Fig. 472.

Fig. 470.—*Leishmania braziliensis.* Parasites in rosette pattern. Smear from an ulcer. (Pessôa in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Fig. 471.—Leptomonas stages in a culture of *Leishmania braziliensis* in N.N.N. medium. (Pessôa in Clinical Tropical Medicine.)

Fig. 472.—*Leishmania braziliensis,* leptomonas stages, found in the pharynx of *Phlebotomus whitmani* naturally infected. (Pessôa in Clinical Tropical Medicine.)

The Montenegro Test.—The Montenegro test is an intradermal allergic reaction. The antigen is a suspension of leptomonas in phenolized saline solution.

Inject intradermally in dosage of 0.1 to 0.2 c.c.

Positive reactions show the formation of a specific papule, which attains maximum development in 48 hours, remains 4 or 5 days, and then regresses and disappears in about 4 days, more or less, according to its intensity.

This test gives about 95 per cent positive results. It may be negative in early cases, but in such cases the parasites can always be found.

Leishmania Tropica (Cunningham 1885, Wright 1903)
Cutaneous Leishmaniasis (Oriental Sore)

The etiological agent of cutaneous leishmaniasis or oriental sore is the *Leishmania tropica.* Morphologically it is identical to *L. braziliensis.*

Geographical Distribution.—Cutaneous leishmaniasis is endemic in Italy, Greece, Spain, Morocco, Algeria, Tunis, Tripoli, and Egypt. It is also found in Abyssinia, Nigeria, French Congo, etc. It is common in Syria, Israel, Saudi Arabia, Iraq, and other countries of Asia Minor, as well as in certain regions of the U.S.S.R., as in the Caucasus and Southern Russia. There are many cases in India and Indochina.

Cultivation.—*L. tropica* can be cultivated in N.N.N. medium. The forms and development are identical with those of *L. braziliensis.*

Transmission.—It is believed that transmission in nature is effected chiefly through the bite of the Phlebotomus, *P. papatasii, P. sergenti,* and *P. caucasicus.*

Diagnosis.—Diagnosis is made by demonstrating the parasite in lesions by smear or by culture, as described above.

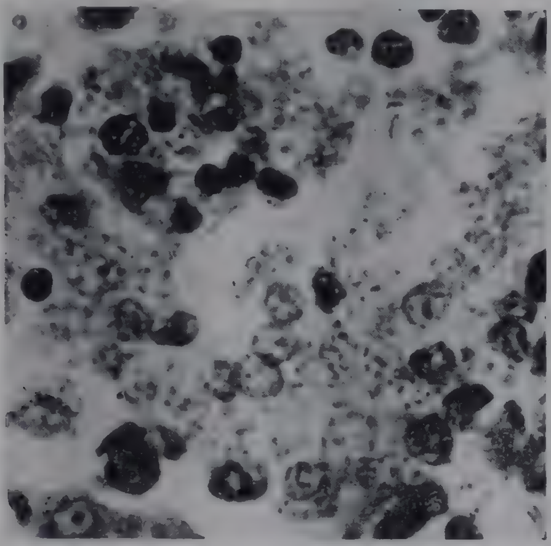


Fig. 473.—*Leishmania donovani* spleen puncture. (O. Felsenfeld, in Clinical Tropical Medicine.)

Leishmania Donovani (Leishman and Donovan 1903)
Visceral Leishmaniasis (Kala-Azar)

The **etiological agent** of visceral leishmaniasis is the *Leishmania donovani.* It is similar in morphology to the *L. braziliensis* and the *L. tropica.*

The disease is known in India as "kala-azar," "black fever," "dumdum fever," or "tropical splenomegaly"; in the Mediterranean as "infantile kala-azar" or "splenic anemia of children"; and in South America as "American visceral leishmaniasis" or "American kala-azar."

Cultivation.—Cultures of *L. donovani* may be obtained from the spleen or from some other organ in which the parasites exist. They have been cultivated from centrifuged urine.

The behavior and morphology of this species in cultures are the same as those of *L. braziliensis.*

Laboratory Diagnosis.—Laboratory methods may be direct, by examination for the parasites, or indirect, by serologic reaction. Microscopic preparations can be used to demonstrate the parasites in the blood, the spleen, the bone marrow, or the liver. These preparations should be fixed in methyl alcohol and stained with Giemsa stain.

The leukopenia may be of great diagnostic value.

Examination of the **peripheral blood** reveals parasites in the leukocytes. Since these are difficult to find, the blood should be diluted with an oxalate solution and immediately centrifuged in a special container. After sedimentation, the layer of leukocytes is drawn off with a pipette. Histiocytes containing many organisms are easily found. A blood culture may be made.

Puncture of the spleen is the method of choice in the search for leishmanias.

To make the puncture, introduce a needle 10 cm. long and 2 mm. in diameter below the costal margin and at a distance which varies with the type of spleen.

Generally parasites are found in smears from the splenic pulp, in great numbers when the case is recent. In the period of the "state of disease," leishmanias are less numerous and, for the most part, extracellular. The pulp may be used to prepare cultures of the parasites.

Resort to **puncture of the liver** is less frequent than splenic puncture, since hepatomegaly occurs later in the disease than does splenomegaly. Finding parasites in the liver is more difficult than finding them in the spleen.

The sternum is usually selected when a search for parasites is to be made in the **bone marrow.** They are frequently found.

Formol-Gel Test (Aldehyde Reaction)*

Place 1 c.c. of the patient's serum, free from erythrocytes, in a 9 mm. test tube. Add 1 drop of 40% (commercial) formaldehyde.

A positive result is indicated by complete coagulation of the serum. Coagulation is complete if the serum does not move when the tube is inverted or shaken. One hour is allowed for the coagulation process.

It has been noted that the serum of a patient with kala-azar becomes coagulated in a few seconds, and not in a few hours.

This test, like other serologic tests, is not absolutely specific. While it is always negative in normal individuals, solidification of the serum takes place in other diseases besides kala-azar: malaria, leprosy, bouba, and alastrim, although the process takes a long time. In American visceral leishmaniasis, the reaction is slow in the early stages, but increases in rapidity with the degree of infection. It is useful in the diagnosis of American visceral leishmaniasis in the early stages and also it can be used for evaluating the results of therapy.

Brahmachari Test (Reaction of Ray)

The Brahmachari test consists of the formation of a turbid ring at the point of contact between the serum and distilled water.

Allow 2 or 3 c.c. of distilled water to flow slowly along the walls of a test tube containing 1 c.c. of serum.

A turbid ring forms at the point of contact in positive cases; the stronger the reaction, the more definite the ring.

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*Spackman, Col. W. C.: Brit. M. J. 2: 266, 1921.

The reaction is due to increase of globulin, principally euglobulin, in the serum, with corresponding reduction in albumin. The reaction is not specific for kala-azar, but occurs as well in malaria.

Complement Fixation Tests

Antigen for complement fixation tests for visceral leishmaniasis are cultures of leishmanias washed in physiologic solutions by centrifugation, and preserved in acetone for a few days. After evaporation of the acetone, the leishmanias which remain are triturated and mixed with ethyl alcohol. Positive results have been obtained with this antigen, not only for visceral and cutaneous leishmaniasis, but also for Chagas' disease. This has been interpreted as the result of a group reaction. This is a delicate test and is not readily adaptable as a routine measure.

Recently antigens prepared from acid-fast organisms have been used for complement fixation tests.

For **intradermal skin tests**, see Mucocutaneous Leishmaniasis.

SUBPHYLUM CILIOPHORA Doflein 1901

Subphylum Çiliophora includes protozoa provided with cilia which serve as organs of locomotion and for taking food. It has two classes, Ciliata and Suctoria.

Ciliophora contain both a macronucleus and a micronucleus. Most of the Ciliophora are free-living, but the class includes a number of parasitic forms.

Class **CILIATA** Butschlii

Order **HETEROTRICHIDA** Stein

Family **Bürsariidae**

All species have cilia or cirri during the vegetative or trophozoite life. Only the genus Balantidium is of interest in medical protozoology.

Balantidium Coli (Malmsten 1857) Stein 1862

The trophozoite of *Balantidium coli* is usually very large. There are also small forms. In the stools, the size varies from 50 to 100 microns in length and 40 to 70 microns in width; some forms are 50 to 80 microns long and 40 to 60 microns wide. The parasite is ovoid and is covered with cilia, which are in constant motion. There are two nuclei, the larger one situated in the midportion of the body, the smaller one usually hidden under the concavity of the macronucleus. A large vacuole is seen in the middle portion and a small vacuole in the posterior end.

B. coli may engulf bacteria, erythrocytes, or other foreign bodies.

Cysts are spherical or ovoid, and large, 45 to 65 microns in diameter, with a thick cystic wall of double contour. It is probable that the cysts which contain the moving trophozoites are viable and that the older cysts are degenerated and dead forms.

Hogs, various species of monkeys, and man can be parasitized by *B. coli*.

The parasites can produce ulcerative lesions in the mucosa of the large intestine.

B. coli has been cultivated on various media.

Man is infected through ingestion of the cysts, either in contaminated food or on the contaminated hands of individuals who slaughter or handle hogs, or by butchers. More than 25 per cent of the reported cases of human balantidiasis give history of direct antecedent contact with hogs.

Diagnosis is made by direct microscopic examination of fresh stools, unstained and stained by Lugol solution. The organisms can be cultivated on a medium composed of 1 part of inactivated human serum and 16 parts of 0.5 per cent saline.

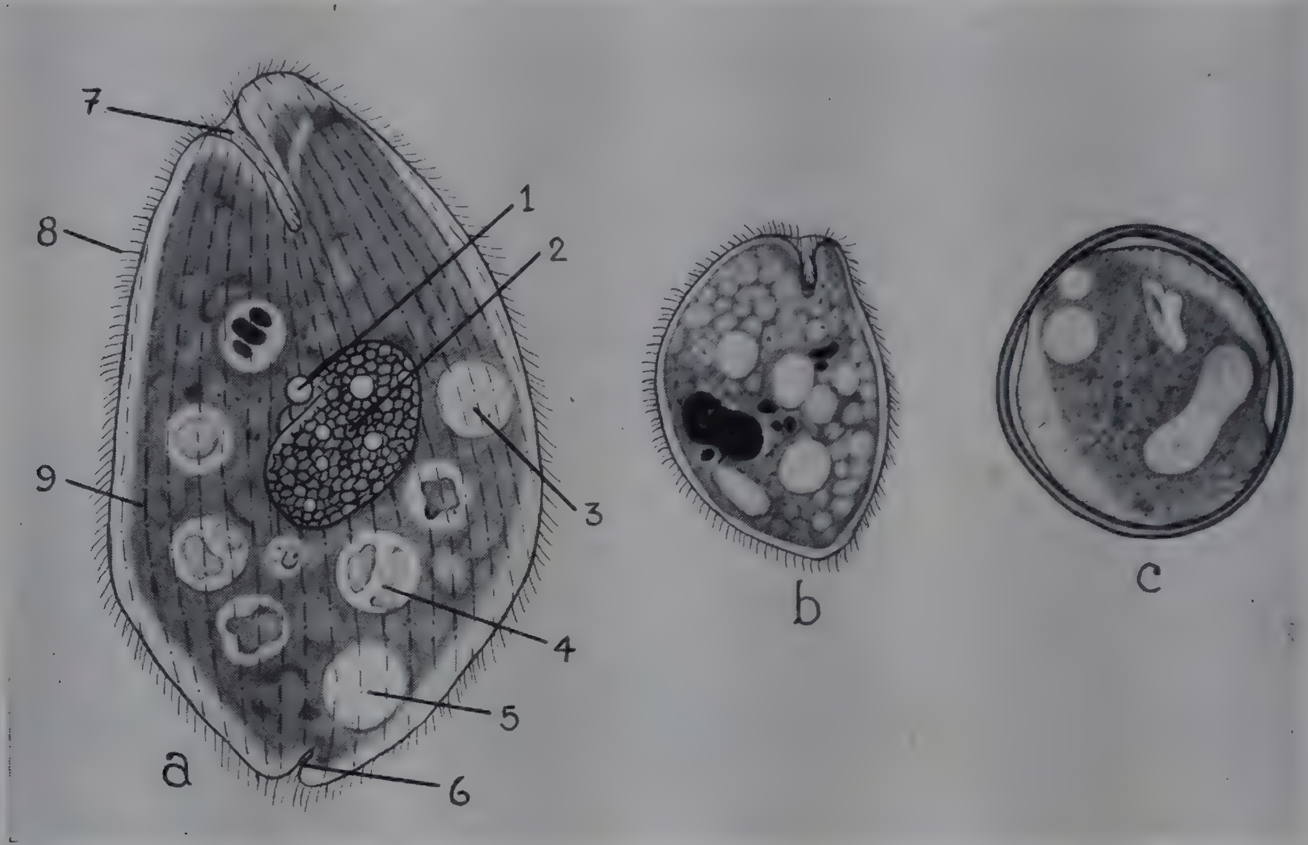

Fig. 474.—*Balantidium coli.* *a,* Living trophozoite in the feces of a pig. Left side. *b,* Trophozoite stained with iron-hematoxylin, from human feces; ventral surface, half the diameter of *a.* *c,* Living cyst in feces of a pig, same diameter as *b.* *1,* Micronucleus. *2,* Macronucleus. *3,* Anterior contractile vacuole. *4,* Nutritional vacuole. *5,* Posterior contractile vacuole. *6,* Anus or cytopyge. *7,* Mouth or cytostome. *8,* Cilia. *9,* Longitudinal stria. (Redrawn from Dobell and O'Connor, in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

FUNGI, SPIROCHETES, AND INTESTINAL BACTERIA

In addition to the protozoan intestinal fauna of man there are frequently seen in the microscopic examination of stools a certain number of intestinal vegetable microorganisms belonging to the classes of fungi, spirochetes, and bacteria, some of which demand the attention of the microscopist. These organisms are of particular importance in teaching students or beginners. Certain elements such as the *Blastocystis hominis* are often sources of error in diagnosis of intestinal protozooses, particularly amebiasis.

Among the fungi the *Blastocystis hominis* must be considered as the principal artefact; in second position are yeasts, monilias, and fungi, often due to external contamination of fecal material. As to the *Spirochaeta intestinalis,* Kourí and Basnuevo[1] published a description of four types based on their morphology in human stools.

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[1] Crón. méd. quir. de la Habana 59: 349, 1933.

Certain bacteria, because of their size, form, and abundance, frequently call attention to their presence under the microscope, for example, the *Clostridium iodophilum*. These organisms are spherical or oval, relatively large, grouped in bunches, refractile in fresh specimens, and blue when stained with iodine. They form a part of the normal flora of the colon. They are very abundant in liquid stools after the use of laxatives which cause rapid emptying of the colon. They are not usually found in the formed stools of normal movements or in the hard stools of constipation.

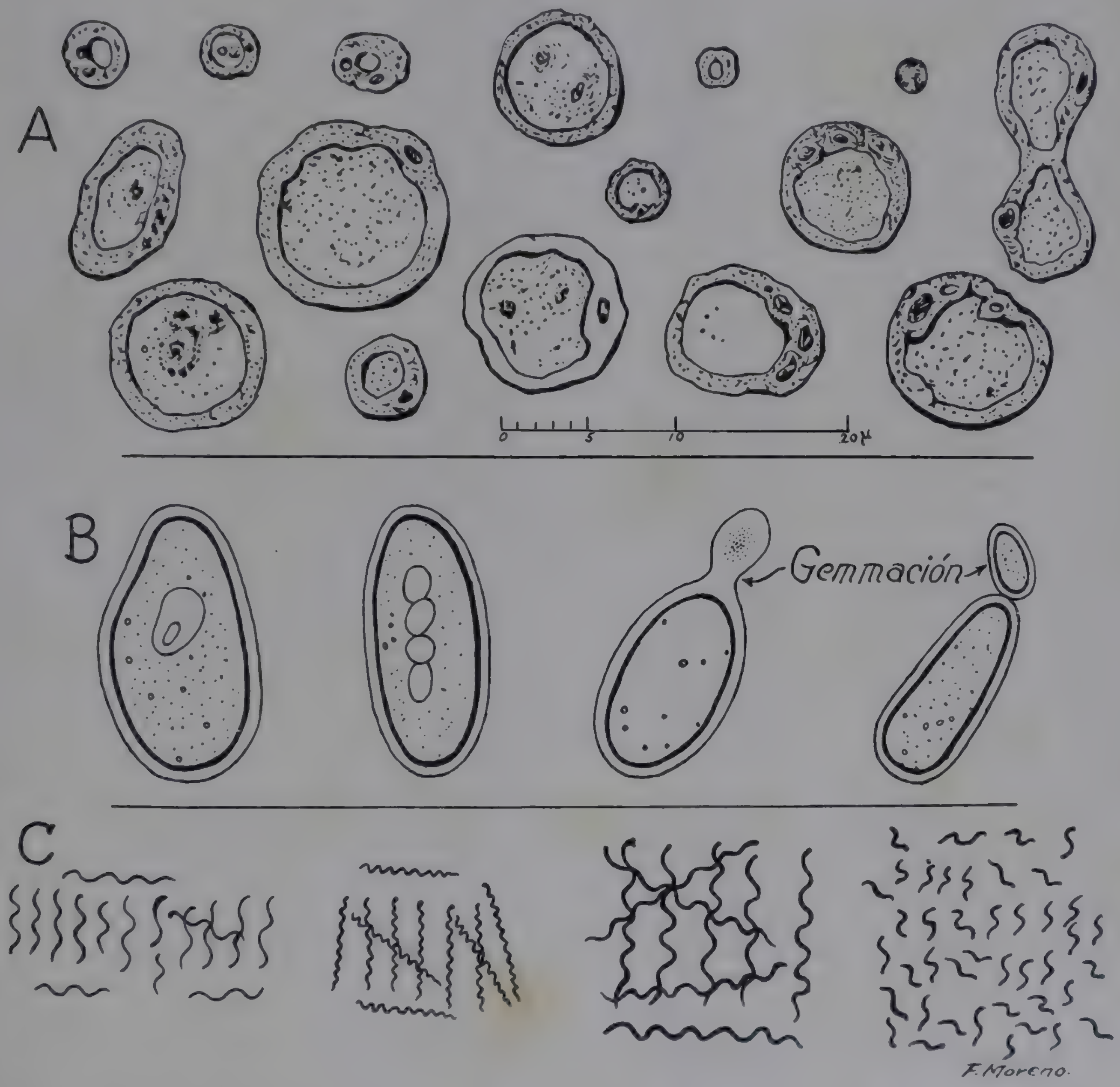

Fig. 475.—*A*, *Blastocystis hominis*, after Langeron and Rondeau du Noyer. *B*, Yeasts, after Troissier and Achalme. *C*, Intestinal spirochetes. All these elements are found frequently in human feces. (After Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

Blastocystis Hominis Brumpt, 1912

Blastocystis hominis appears in the stools as a spherical body, colorless and refractile, varying in size from 2 to 15 microns in length (see Fig. 475). In some specimens only the small forms are seen, while in others medium-sized forms are found, and in still others, the very large forms. The small forms are frequently confused by beginners with cysts of amebae.

When stained with Lugol, the characteristic structure can be seen very well. It has a relatively thick peripheral ring of protoplasm in which may be seen one or more nuclei with dense karyosome, and a central or slightly excentric vacuole. It stains well by Gram stain (Kourí 1930) and by iron-hematoxylin. At times they may be seen in binary fission, biscuit-shaped, or in figure of eight. They are frequently seen in individuals with slight intestinal disturbances, or even with no such disturbances. They are seen at times in considerable numbers in diarrheal or dysenteric stools, often associated with true intestinal protozoa and spirochetes.

It is usually regarded as nonpathogenic but when seen in large numbers in diarrheal or dysenteric states, alone or associated with spirochetes, in patients in whom it is otherwise impossible to ascertain any other cause for the disturbance—a disturbance which disappears with the disappearance of the Blastocystis when pentavalent arsenicals are administered by mouth—there is a possibility that it may be able to cause such symptoms, or that it has contributed at least in their production or maintenance. In other words, in such cases the *Blastocystis hominis* might be the cause and not the consequence of these symptoms, especially when they occur in great numbers.

Yeasts, Monilias, and Other Fungi in Human Stools

Yeasts are oval, mammilated elements, hyaline and refractile, with a diameter not exceeding 6 to 7 microns. They are frequently found in stools. When found in large quantity they are generally due to ingestion of brewer's yeast. These yeasts must not be confused with Clostridia which are also oval, but which are much smaller and which stain very light blue with Lugol. True yeasts take on the yellow color of Lugol. Finding of yeasts in stools concentrated by Telemann's method is quite common. Most of them are gram-negative. See Fig. 475.

Other fungi found in stools are the Monilias, which appear as filamentous forms composed of segments with rounded extremities, distended and very fragile, with an oval blastospore like the yeasts, but generally wider and more easily broken off. In cultures of stools on Sabouraud's medium, monilias and yeasts frequently develop. Stools that have been exposed to the air are frequently contaminated by fungi from outside the body. Do not consider these fungi as parasites coming from the intestinal canal.

Intestinal Spirochetes

Different types of spirochetes are found frequently in stools. They are found in large numbers in diarrheal conditions, at times in dysenteric syndromes, alone or associated with Blastocystis or other intestinal protozoa.

SPOROZOA

Coccidiosis of Man

Coccidia are protozoa which are widely distributed usually in the animal kingdom. They show a complicated development, and multiply by sexual and asexual methods. They have been found in the stool in cystic form, the so-called oöcysts.

Eimeria stiedae is a rabbit coccidium which has been reported in a number of cases in man. Silcock, of London, in 1890 reported a fatal case in man with intestinal disturbances, anemia, and fever, showing at autopsy a marked enlargement of the liver with numerous ulcerative caseous areas filled with coccidia. Dresler and Craig report a case with liver nodules filled with coccidia.

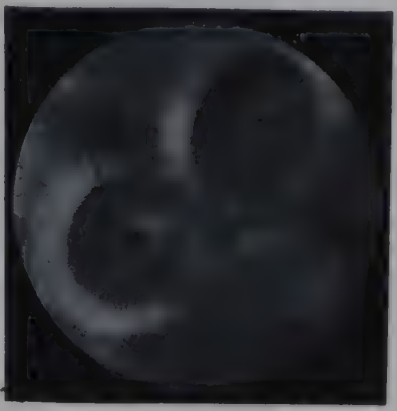

Fig. 476.—Coccidiosis of man, *Isospora bigemina* (×950).

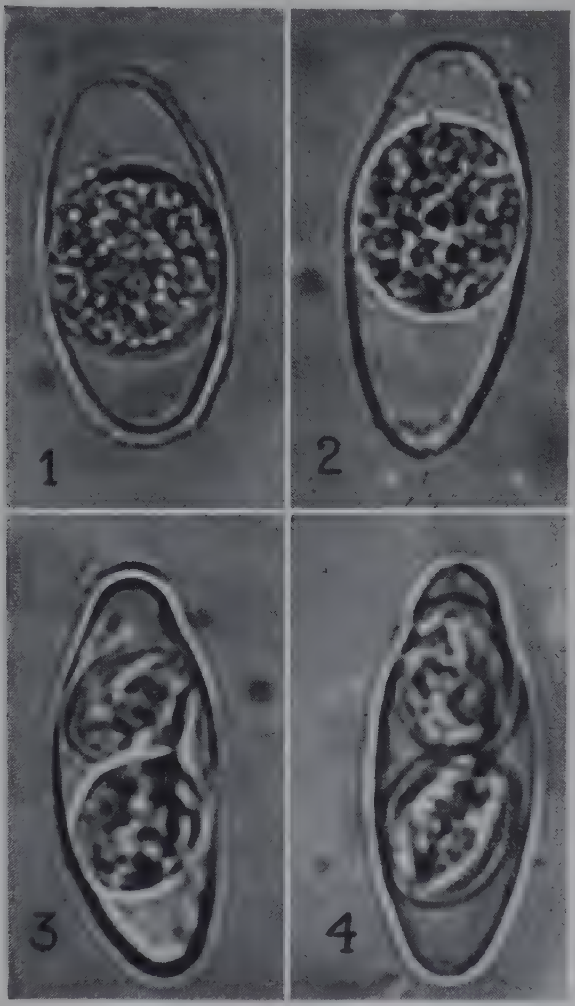

Fig. 477.—Human coccidia: *Isospora hominis.* *1* and *2*, Oöcysts with one sporoblast, in a concentrated specimen, method of Willis; fresh feces recently passed. *3* and *4*, Oöcysts with two sporocysts, from a 48-hour culture of fecal material concentrated by the method of Willis. Each sporocyst produces four sporozoites. Thus, 2 sporocysts × 4 sporozoites = 8 sporozoites. (After Kourí and Basnuevo in Revista de Medicina Tropical y Parasitología, 1937, and Lecciones de Parasitología y Medicina Tropical.)

Isospora Hominis.—These cases have shown the typical oöcysts in the stool. These oöcysts were found in conjunction with a dysenteric condition. There has also been described in man as well as in cats and dogs a special type of isosporocyst known as the *Isospora bigemina.* Reichenow, with whom the author worked at the Hamburg Institute of Tropical Medicine, presented him with the specimen which is photographed here. (Fig. 476.)

Eimeria Oxyspora Dobell, Eimeria Wenyoni.—In these cases we find the characteristic Eimeria oöcysts in the stool of the infected patient. The *Eimeria wenyoni* seems to be identical with the *Eimeria clupearum* which has been recovered from the liver of herring and other fish.

TOXOPLASMOSIS

Toxoplasma Gondii (Nicolle and Manceaux)

Many reports on toxoplasmosis have appeared since the original discovery of toxoplasma by Nicolle and Manceaux, in 1908, in North Africa, and by Splendore in the same year, in Brazil. Nicolle and Manceaux first observed intracellular parasites in smears of the spleen and other organs of the North African rodent, the gondi (*Ctenodactylus gondi*). They were impressed by its resemblance to Leishmania. They recognized the absence of a kinetoplast and later, in 1909, placed the parasite in the new genus Toxoplasma. They described it fully, even to the point of recognizing its mode of division as longitudinal bipartition. In accordance with the laws of priority for binomial nomenclature, the proper name for all strains of Toxoplasma is *Toxoplasma gondii* (Nicolle and Manceaux).

With the first strain they isolated, they were successful in producing acute toxoplasmosis in the gondi and the guinea pig, but failed with macaques and rats. Splendore, in 1909, reported that he was able to transmit the parasite to guinea pigs and to sparrows and other birds. Carini, in 1909, was able to infect pigeons with the parasite from rabbits, as well as guinea pigs and white rats, but not dogs or chickens. Nicolle and Conor (1913) found mice to be most susceptible, but they failed to infect white rats, a young dog, monkeys, frogs, or toads. Mello, in 1910, in the meantime described a spontaneous case of toxoplasmosis in a dog, and Carini, in 1911, and Carini and Maciel, in 1913, also discovered natural infections in dogs and pigeons and transmitted the parasite to other animals. Carini and Migliano, in 1916, found natural infection also in guinea pigs from which they inoculated pigeons successfully. Chatton and Blanc, in 1917, were able to infect a cat with a parasite from the gondi. It thus became clear that Toxoplasma was a parasite without host specificity among warm-blooded vertebrates, and Chatton and Blanc expressed the view that the organisms found naturally in the various hosts were all members of the same species. This was substantiated on immunologic grounds as well by Sabin in 1939. The discrepancies in some of the early reports regarding the susceptibility of various animals to Toxoplasma may be attributed to variations in host response and in strain virulence.

Leon Jacobs[1] discussed the biology of toxoplasmas fully. Toxoplasmosis has been found in the gondi, the mouse, the vole, rat, rabbit, guinea pig, squirrel, and marmot. Among carnivores, the dog, cat, and chinchilla have been found naturally infected. There are also reports of infections in sheep and in swine. Among primates, it has been found in the baboon and chimpanzee as well as in man. Forty-five species of birds have been reported as harboring this parasite. The pigeon has been definitely shown to harbor Toxoplasma.

Morphology.—Toxoplasma resembles the merozoite of a coccidian. It is crescent-shaped, with one end attenuated, the other more rounded. It is 2 to

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[1] Am. J. Trop. Med. & Hyg. 2: 365, 1953.

4 microns wide by 4 to 7 microns long. The nucleus is nearer the rounded end. No centrosome or kinetoplast is visible, but in some specimens there may be a chromatic granule between the nucleus and the attenuated end. At times the parasite appears oval rather than crescentic, and this stage is considered preliminary to cell division. No pigment is present in any stage. The organism stains well with Wright or Giemsa stain, with a pale blue cytoplasm and a red nucleus.

Agglomeration of the parasites within muscle cells has given rise to reports that the Toxoplasma reproduces by schizogony as well as by longitudinal division, but no intermediate stages representative of schizogony have ever been described.

Culture.—Toxoplasma requires an intracellular situation to grow and multiply. Sabin and Olitsky, in 1937, pointed out that as an obligate intracellular parasite it is similar to a filtrable virus in behavior and in the host reactions it induces. It has never been cultivated in artificial media, but it has been propagated successfully in suspensions of minced embryonic tissues by Sabin and Olitsky (1937) and in Maximow slide cultures of chick embryonic tissues by Guimaraes and Meyer (1942) and by Jacobs.

Inoculation into the yolk sac of a developing chick results in dissemination of the parasite, which can be recovered from chorio-allantoic and other membranes and from the embryonic tissues in one week or less. The virulence of the parasites following over 13 serial passages in chick embryos remains unchanged, and Jacobs states that this may be extended to include a much longer period, two to three years. He also states that a chick embryo appears to be a good host in which to maintain Toxoplasma strains of low virulence.

Transmission.—Intracerebral inoculation of Toxoplasma into mice, rabbits, hamsters, guinea pigs, pigeons, and other hosts generally results fatally within one week, even with Toxoplasma strains of low virulence, and in hosts such as the rat which manifests little evidence of infection following peripheral inoculation even with virulent strains.

Pathogenicity.—It appears that neonatal toxoplasmosis could result from transfer to the child of toxoplasmas in milk of mothers experiencing the initial acute stage of the infection after parturition. It is unlikely that this could occur in chronic infections. On the basis of clinical and laboratory findings, the likelihood of congenital transmission of toxoplasmosis from the chronically infected mother is low.

According to Sabin,[2] the cardinal characteristics of Toxoplasma are (1) typical morphology and staining characteristics of extracellular and intracellular forms in impression films of fresh tissue or exudate; (2) pathogenicity for mice, guinea pigs, rabbits, chicks, etc.; (3) obligate intracellular parasitism; (4) immunologic relationship with established strains.

Unequivocal proof that toxoplasmas are the cause of certain disease conditions in human beings was first obtained in 1939. The toxoplasmic etiology of certain types of congenital encephalomyelitis and ocular damage, of certain types of acute encephalitis in older children, of a spotted-fever-like disease and pneumonitis in adults now rests on the firm foundation of demonstration of the organisms in the tissues.

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[2] Am. J. Trop. Med. & Hyg. 2: 360, 1953.

Although toxoplasmosis has been suggested as a possible cause of spontaneous abortions of unknown etiology, it is probably not a common cause. Feldman and Eichenwald together performed serologic tests on 95 women with spontaneous abortions of unknown etiology and found no evidence of current toxoplasmosis.

Studies by Siim in Denmark revealed that among patients with unexplained febrile illnesses associated with lymphadenopathy, a certain number exhibited serologic evidence of recent infection with Toxoplasma. Pinkerton found morphologically typical toxoplasmas in the lymph node of an American woman with a high titer of dye test antibody. Feldman, Eichenwald, and Jacobs found no evidence of active or recent infection with Toxoplasma in more than 100 patients with acute, active, or recurring chorioretinitis tested by the new serologic methods.

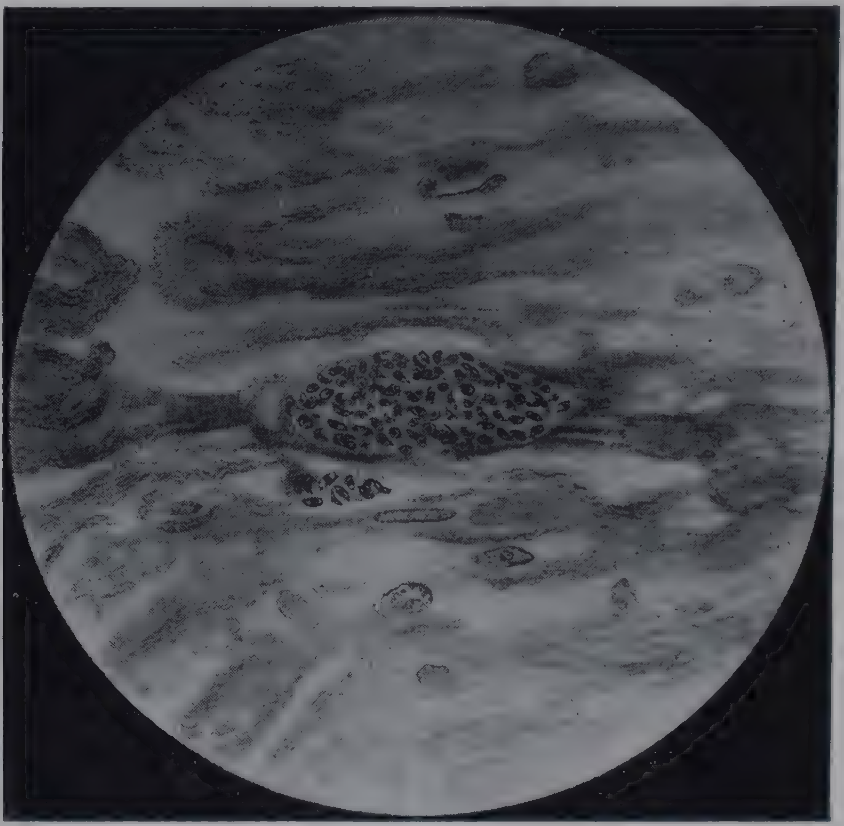

Fig. 478.—Toxoplasma in section of cardiac muscle of a dog. Drawing by David Eichelson of the N.I.H. staff. (Courtesy Leon Jacobs, Laboratory of Tropical Diseases, National Microbiological Institute, National Institutes of Health. Am. J. Trop. Med. & Hyg. 2: 365, 1953.)

It seems to be a fact that countless millions of people in various parts of the world become infected with toxoplasmas during their lifetimes. If this is true, perhaps the clinically recognizable disease associated with such widespread infection might not be as rare as it now seems to be; future awareness and intelligent application of appropriate diagnostic tests might supply us with clues to clinical syndromes of toxoplasmosis which are now lost in the vast mass of undiagnosable human disease.

MALARIA

Order **HAEMOSPORIDIA** Danilewsky

The order Haemosporidia includes small sporozoa and usually intracellular parasites of erythrocytes as well as those found in the endothelial cells of the vertebrate host.

Family **PLASMODIDAE** Mesnil 1903

The Family Plasmodidae includes species which produce pigment within erythrocytes of vertebrates and also produce gametocytes. Species of the genus Plasmodium cause human malaria.

Malaria is a disease caused by protozoa belonging to the genus **Plasmodium.** The parasite is found in the red blood cells of the circulating blood. Malaria produces periodic febrile attacks leading later to high-grade anemia, swelling of the spleen, and severe cachexia.

The geographic distribution is very extensive. An area of about 30° latitude on each side of the equator may be considered as the malarial belt which encircles the earth. Malaria is found in tropical and temperate zones and as far north as 65° latitude in Europe and Asia and about 40° north latitude in North America. Where malaria is not found it is due to the absence of the insect vector or to a mean temperature not greater than 15° C. (59° F.). Approximately 10,000,000 cases have been reported in India, and possibly that number abounds in Africa. It is not definitely settled whether the disease was brought to the New World from Africa in slave ships or whether it was found in the Americas at the time of the discovery by Columbus.

The cause of malaria was discovered in 1880 by Laveran, in Algiers. Richard, in 1882, was the first to confirm Laveran's discovery.

Malaria and Mosquito

In 1896, Manson announced his theory that mosquitoes die within a few days after they have laid their eggs upon the surface of the water. He thought then that the drinking of such water by man resulted in infection, but earlier attempts to infect man by drinking water from swamps absolutely failed. The true story of the sexual development of this parasite in the mosquito was theoretically worked out by Manson and actually proved by Ross in 1897, later confirmed by Grassi. Manson communicated his theoretical idea to Ronald Ross, who, at that time, was a young officer in the medical corps of the British Army. Ross had a long service with the Colonial Forces in India and was a student of mosquitoes even before he had any idea of their role in malaria. While on a visit to England, he was instructed by Manson in the appearance of the malarial parasite and returned to India determined to put Manson's theory into practice. He definitely proved Manson's ideas to be correct. He returned to India in 1895. In 1897 he had successfully proved the role of the mosquito as the intermediary infecting agent in malaria. Four years of hard work followed, studying mosquitoes. He noted the two varieties, the "gray" (Culex) and the "black-striped winged" variety (Stegomyia). On Aug. 20, 1897, in Secundcarabad, India, Ross demonstrated that the sporozoites of the malarial parasites were seen in the stomach wall of "large dapple-winged mosquitoes," and that these were undoubtedly of the Anopheles variety. These mosquitoes were cultivated from larvae and were inoculated with crescent parasites found in blood.

In the same year MacCallum (1897-1898) observed the fertilization of the female gametocytes by the male.

Ross noted that five days after the initial infection the pigmented cells in the mosquito stomach wall were filled with partially developed sporozoites.

This constituted the beginning of the development of the malarial parasite in the mosquito. In 1898 Ross showed the whole process of development of the organism, using bird malaria, or Proteosoma, in the Culex mosquito. He also proved the carrying over of malaria of birds from an infected mosquito to healthy birds.

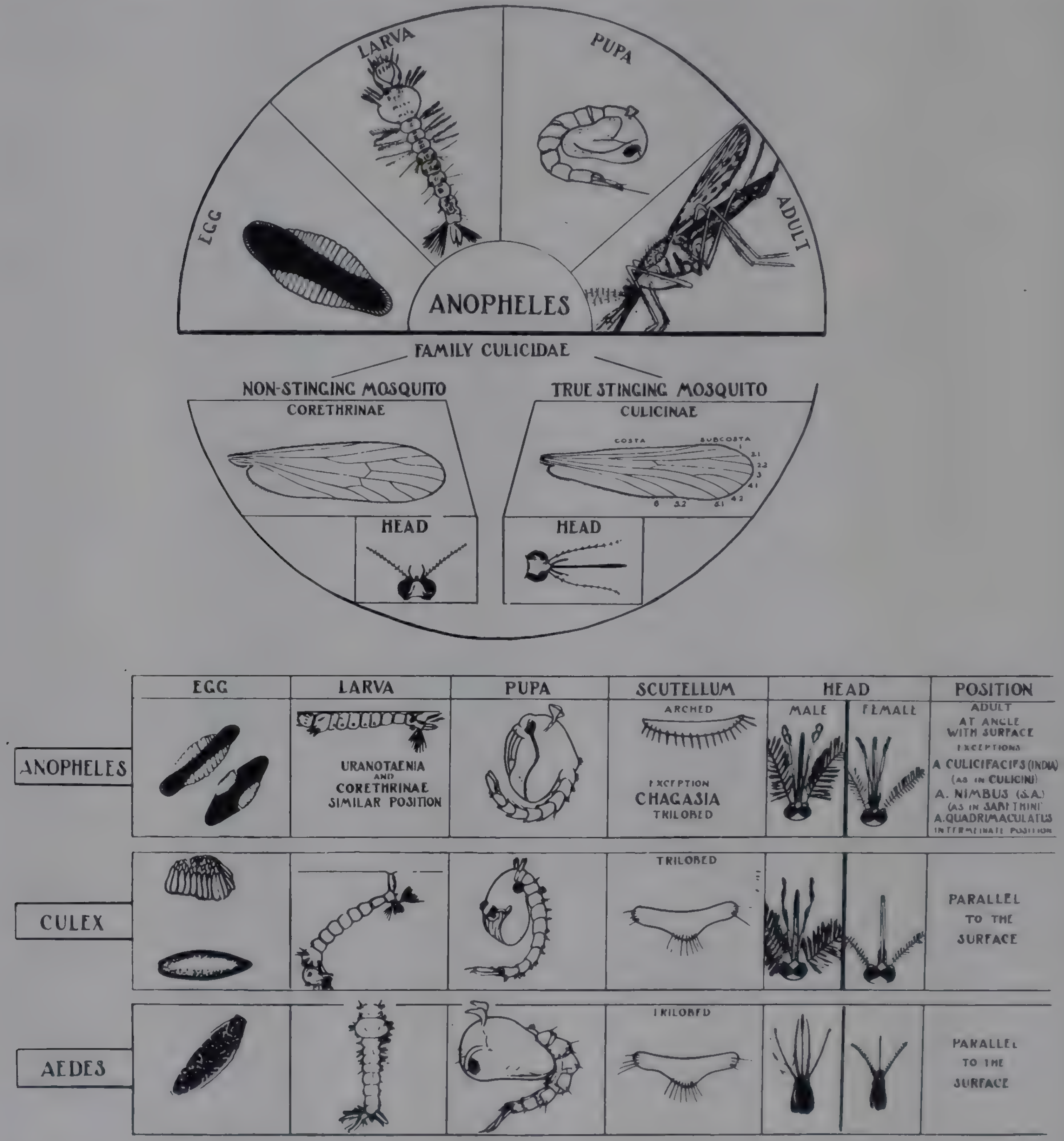
EGG
LARVA
PUPA
ADULT
ANOPHELES
FAMILY CULICIDAE
NON-STINGING MOSQUITO
CORETHRINAE
TRUE STINGING MOSQUITO
CULICINAE
COSTA
SUBCOSTA
HEAD
HEAD
EGG
LARVA
PUPA
SCUTELLUM
HEAD
MALE
FEMALE
POSITION
ANOPHELES
URANOTAENIA AND CORETHRINAE SIMILAR POSITION
ARCHED
EXCEPTION
CHAGASIA
TRILOBED
ADULT AT ANGLE WITH SURFACE
EXCEPTIONS
A CULICIFACIES (INDIA) (AS IN CULICINI)
A. NIMBUS (S.A.) (AS IN SABETHINI)
A. QUADRIMACULATUS INTERMEDIATE POSITION
CULEX
TRILOBED
PARALLEL TO THE SURFACE
AEDES
TRILOBED
PARALLEL TO THE SURFACE

Fig. 479.—Differentiation of mosquitoes. (Chart planned and drawn by Dr. Alfonso Bonilla Naar, of Bogotá, Colombia, and published through his courtesy by permission of copyright owner, General Biological Supply House, Chicago, Illinois.)

Grassi, Bignami, and Bastianelli in 1898 confirmed this work. Bignami in 1898 was the first to carry over true malaria through an infected Anopheles mosquito. Grassi, Bignami and Bastianelli, toward the end of 1898, showed the full development of the tropical as well as the tertian and quartan parasites in the blood of the *Anopheles claviger*. Ross in 1899 showed further that all three parasitic varieties could be developed in *Pyretophorous costalis* or *Myzomyia funesta* like the proteosoma in *Culex fatigans*. Grassi in 1899 and other Italian workers, and Ziemann in 1900, showed that the Anopheles is the only type of mosquito to carry the human parasites.

Attempts were made by Mesnil and Rouband in 1917 to carry over the malarial parasite to chimpanzees. Reichenow in 1917 and 1920 in the Cameroon attempted the inoculation of anthropoid apes with human malarial parasites. These attempts were unsuccessful.

Malaria is transmitted through the bite of the anopheline mosquito. The known vectors in North America are *Anopheles quadrimaculatus* (a good vector), *A. crucians* (probably a good vector in some sections), *A. maculipennis* (principal vector in Europe and probably also in California), *A. punctipennis* (not known to be a vector till 1916, undoubtedly a poor vector), and *A. pseudopunctipennis* (not known positively to be a vector in North America, but recently shown to be the main vector in parts of Argentina).

It has been proved by Rozeboom and Laird[1] that the *A. bellator* is a vector of malaria. This work was carried out during the 1942 military emergency in Trinidad, in connection with the control of malaria incident to the construction of an Army base at that point. In Trinidad, the control problem had been complicated by the fact that the distribution of malaria could not be explained on the basis of the two known vectors, *A. albimanus* and *A. aquasalis*. It was found that the *A. bellator* breeds in water which collects in the air plants which grow in the Immortelle trees, used locally on plantations as shade for the cocoa trees. It has been suspected that this mosquito was an important vector of malaria on epidemiologic grounds but this was not supported by experimental evidence until the work of Rozeboom and Laird was completed. They showed that the *A. bellator* attacked man viciously, literally swarming about people during the hours when the females became active, and that it attacked man throughout the afternoon, especially on dark days and in the shade of the forest. Experimental infections of *A. bellator* were obtained twice, and proved that the species was very susceptible to at least one of the species of Plasmodium causing human malaria. Oöcysts were found in two of four *A. oswaldoi* that fed on a *P. vivax* gametocyte carrier. Three of 725 ''wild'' *A. bellator* were found to be naturally infected with Plasmodium. The evidence collected by Rozeboom and Laird confirmed the conclusions of DeVerteuil[2] that *A. bellator* is the vector of malaria in the interior cocoa growing districts of Trinidad.

The Anopheles belong to the subfamily Culcinae. The Culcinae are divided into two tribes—Anophelini and Culicini. The tribe Anophelini has been divided into a large number of genera (about thirty-eight) but it has been proposed to recognize only one genus, Anopheles. It is important to distinguish between the Anopheles and the Culex, which is the ordinary nonmalaria-carrying mosquito. All the anophelines can be distinguished by the palpi, which in both the females and males are as long as the proboscis. The female of no other species of mosquitoes has such long palpi. The larvae, when feeding, float parallel to the surface of the water and do not possess a respiratory siphon.

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[1] Rozeboom, L. E., and Laird, R. L.: Am. J. Trop. Med. 22: 83, 1942.

[2] DeVerteuil, E. J.: 1935 Administration report of the Surgeon-General for the year 1934. Government Printing Office, Port-of-Spain, Trinidad.

Plate XLIX.—Malarial Parasites.

Plasmodium vivax

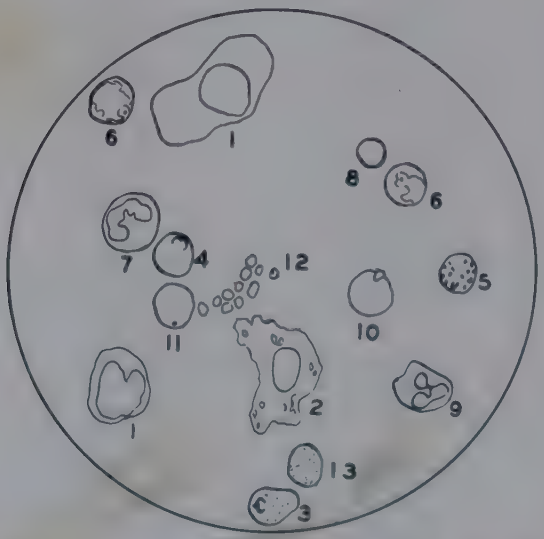
1 and 2. Ring forms.
3 and 4. Early ameboid forms.
5. Double ring form.
6. Ring form.
7, 8, 9, 10. Ameboid forms.
11. Microgametocyte
12. Macrogametocyte.
13 and 14. Ameboid forms.
15. Ameboid form showing Schueffner punctation.
16, 17. Division forms.

Plasmodium malariae

18, 19. Ring forms.
20, 21, 22, 23, 24, 25, 26, 27. Ameboid forms showing malaria pigment.
28. Beginning division.
29. Macrogametocyte.
30. Ameboid form showing malaria pigment.
31. Division form.

Plasmodium falciparum

32. Ameboid form showing malaria pigment.
33, 34, 35, 36. Ring forms.
37. Double rings.
38. Section of brain capillary showing malaria pigment.
39. Macrophage showing malaria pigment.
40. Microgametocyte.
41. Double rings.
42. Macrogametocyte.
43. Microgametocyte.
44. Macrogametocyte.
45. Multiple ring forms.

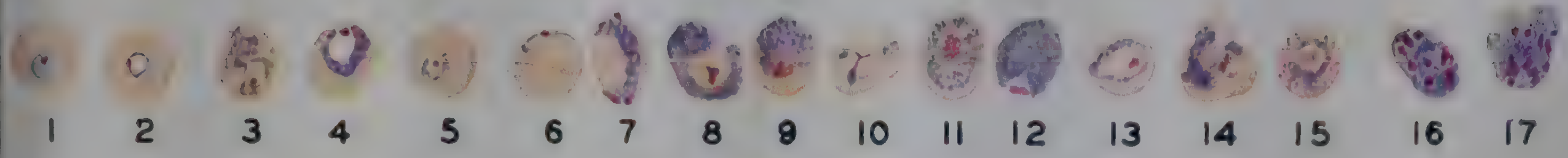


Blood Picture of Malaria Showing Phagocytosis.

(1) Monocyte. (2) Malaria phagocyte. (3) Ring form with Schueffner punctation. (4) Ring form. (5) Division form. (6) Ameboid forms. (7) "Stab." (8) Lymphocyte. (9) Segmental neutrophile. (10) Blood platelet on an erythrocyte. (11) Marginal granule. (12) Blood platelets. (13) Erythrocyte with basophilic punctation.

MALARIAL PARASITES

PLASMODIUM VIVAX (MALARIA TERTIANA)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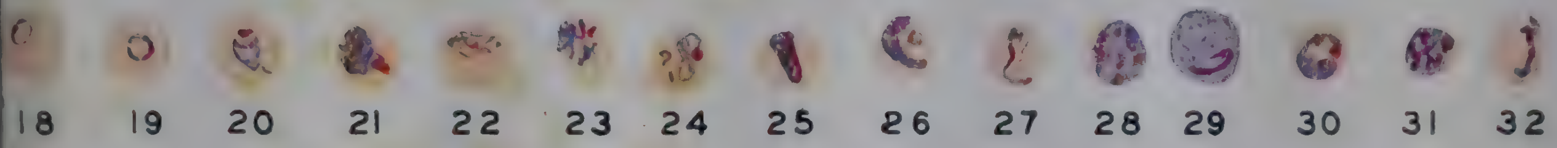
PLASMODIUM MALARIAE (MALARIA QUARTANA)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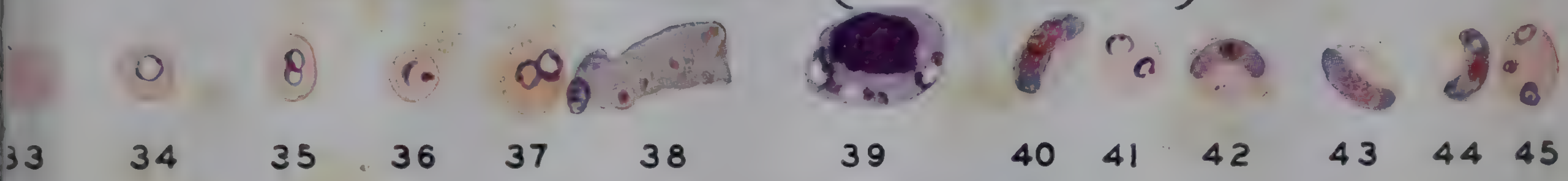
PLASMODIUM FALCIPARUM (MALARIA TROPICA)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BLOOD PICTURE OF MALARIA SHOWING PHAGOCYTOSIS

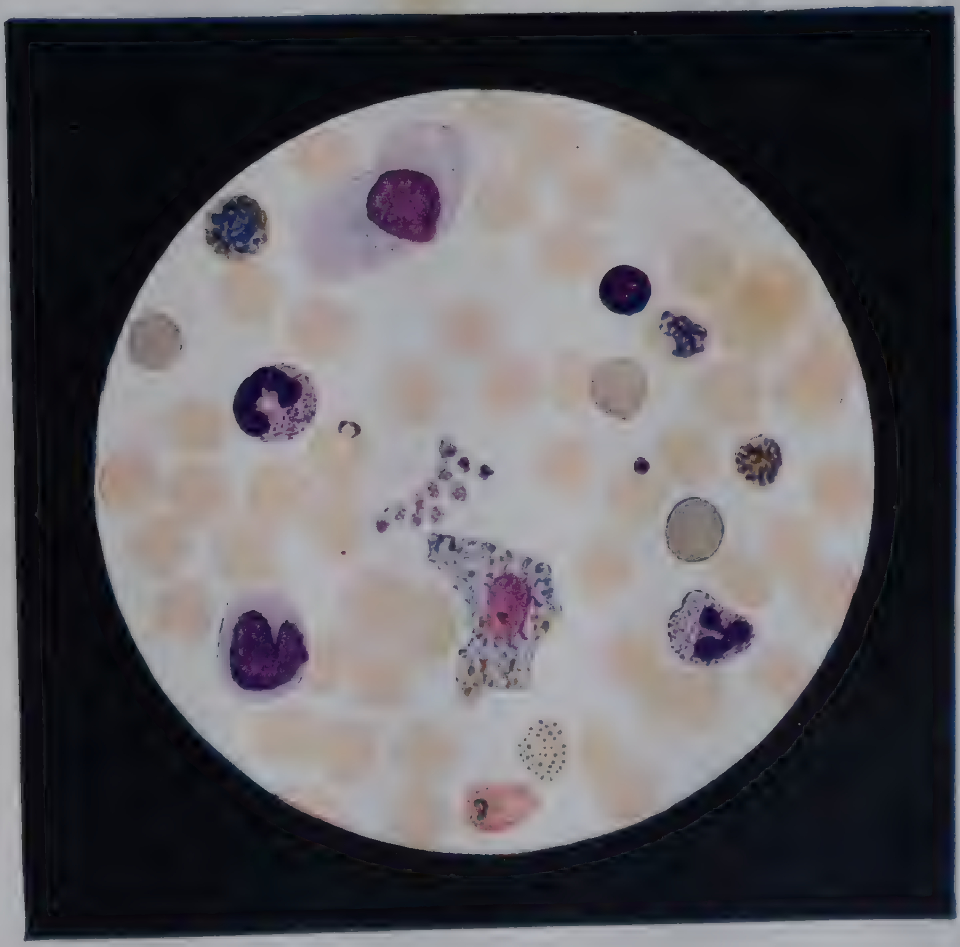

PLATE XLIX.

(See legend on opposite page.)

The winged mosquitoes, Anopheles and Culex, differ from each other in their positions upon the wall. The female Culex sits on the wall with its abdomen parallel and close so that there is a very slight angle. The Anopheles sits with a very acute angle, 50 to 80°.

The female Anopheles have palpi which are long as their stickers, while the Culex have very short palpi. The male mosquitoes of both types have feathery antennae, which readily identify them as males.

The differences in appearance of the two varieties are well seen in Fig. 479.

A. walkeri (Theobald) may confuse malariologists, according to Bellamy and Andrews,[1] who record that more than 80 larval *A. walkeri* were identified in the collections made between Sept. 15 and Nov. 11, 1937, from seven different permanent or semipermanent shallow ponds in southeastern Sumter County, Ga. All the ponds supported heavy growths of emergent vegetation, supplemented in several instances by submerged and floating vegetation and considerable debris.

Twenty adult female *A. walkeri* were taken with collecting tubes while biting between 9:30 and 11:30 P.M., October 5, within 50 feet of a productive breeding place. They found that all of the wild-caught females, plus five raised from larvae, resembled the Florida specimens, having quite distinct bands of white scales on the palpi. Two males which were reared appeared to be similar to *A. quadrimaculatus*, though they were perhaps somewhat darker.

Habits of the Anopheles.—Anopheles are all night-flying insects. The *A. maculipennis* begin to swarm and bite when the sun goes down. With the rise of the sun, they seek dark places. They are most active during twilight. In the tropics, they are very prevalent in cloudy weather with no wind. During the day, they are in the grass and in the dark corners of buildings or in the stables. They avoid wind and rain. A severe rain will throw most of them down and kill them. As soon as it begins to rain, they fly to the houses and huts.

Malarial Parasites

Genus **Plasmodium** Marchiafava and Celli 1885

There are four different varieties of plasmodia which not only are morphologically different, but also produce different and distinct clinical symptoms. There are, however, mixed infections in the same individual. The four species are: (1) *Plasmodium vivax,* the cause of tertian malaria; (2) *Plasmodium malariae,* the cause of quartan malaria; (3) *Plasmodium falciparum,* the cause of tropical or estivo-autumnal, or falciparum malaria; (4) *Plasmodium ovale,* the cause of a benign malaria.

Life Cycle of Malarial Parasites

There are two hosts concerned with the development of the Plasmodium of malaria. One is the vertebrate host in which the cycle is asexual, the other is the invertebrate host, a mosquito, in which the cycle is sexual. The vertebrate and invertebrate hosts have been referred to, respectively, as "intermediate" and "definitive." Some believe that the use of these terms is erroneous. The sexual cycle is initiated in the vertebrate host by the forming of gamete-producing cells. The aggregate of the parasites living within the vertebrate host more nearly corresponds with the adult sexual individual among higher animals since they produce the gametes. A large portion of sporogony appears to be an asexual reproduction. It appears unjustifiable to continue in use the common practice of making alternation of generation strictly parallel without alternation of hosts. Some authors regard the use of the terms "intermediate" and "definitive" hosts for the vertebrate and invertebrate hosts, respectively, as highly questionable. It would seem that the terms "vertebrate phase" and "mosquito or invertebrate phase" for these portions of the cycle would be more appropriate in the present state of our knowledge.

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[1] South. M. J., p. 797. July, 1938.

The great ease with which the parasites can be studied in the mosquito has led to a much more complete understanding of the morphologic development or stages of the parasites in that host than the stages in the vertebrate hosts. At the present time, a connected story of the development of malarial parasites is known only in a few of the species parasitizing birds, but it is known that the development of the parasites of man in the anophelene mosquito, of those of birds in the culicine mosquitoes, of Haemoplotus in the hippoboscid flies, and of Leucocytozoon in simuliids are similar to general outline.

The Invertebrate Phase.—In the Anopheles, when *P. falciparum* is ingested, the blood from the vertebrate host may contain asexual stages of the parasite in addition to sexually differentiated gametocytes. The former are digested in the stomach or midgut of the mosquito. The **gametocytes** undergo gametogenesis in the lumen of the stomach, resulting in transformation into or production of large **macrogametes** and threadlike **microgametes**. These gametes fuse in fertilization, which results in a **zygote,** which is a vermicular **oökinete,** which migrates through the stomach wall and rounds up to form an **oöcyst.**

Lying between the columnar epithelium and the membrane, these oöcysts grow rapidly, increase in size, and undergo complex internal change. These changes involve the repeated division of the nucleus, the formation of many vacuoles which become confluent, the migration of the nuclei to the cytoplasmic surface next to the vacuoles, and the budding off of small portions of cytoplasm, each containing a nucleus.

These budded portions are called "**sporozoites.**" They fill the oöcyst and become arranged in a haphazard manner prior to the bursting of the oöcysts. When this happens, that is, when the oöcyst bursts, the sporozoites enter the hemocele of the mosquito and circulate. No further development occurs in the mosquito. Many of these sporozoites reach the salivary gland, the cells of which they penetrate. Some disappear. The sporozoites then enter the salivary ducts of the mosquito and are deposited in the subcutaneous tissues of man when saliva is injected during the blood-sucking process.

The Vertebrate Phase.—Following Schaudinn's claim in 1903, it has generally been believed that sporozoites of Plasmodia enter directly into red blood cells. It was, for a long time, assumed that all of the schizogony was confined to circulating cells in species belonging to this genus. It is now known that some species of plasmodium have **exoerythrocytic stages of schizogony.** Schizogonic development and metagonic development occur in all types of plasmodia infecting man. This schizogony is characterized by repeated nuclear division, by extensive vacuolization, and by eventual production of merozoites. In most species, schizogony must be repeated many times. This schizogony may take place in an individual host for as long as eight years. In the case of one strain of avian malaria, it has continued without intervention of sexual reproduction for 32 years.

With **erythrocytic schizogony,** the best known stages of malarial parasites which develop in the vertebrate host are of those that live in the erythrocytes. The youngest stage found in the erythrocyte is the uninucleate trophozoite which consists of cytoplasm and a nucleus. In the youngest stage, it is most frequently round or oval. No pigment is visable at this stage. The trophozoite grows at the expense of the erythrocyte and assumes a more variable form which differs widely with the species, but in most cases the formation of pseudopodia is seen, and when observed in the living stage, granules, which vary according to the species, appear soon after the first perceptible growth has occurred. These granules consist of hematin which is formed as the result of digestion of the hemoglobin of the erythrocyte.

The growing parasite is destined to produce either a schizont or a gametocyte. Parasites destined to grow into segmenting forms are schizonts from the beginning of their development. As the parasite grows, further division of the nucleus takes place, a process termed ‘‘**schizogony.**’’ After the last nuclear division has occurred, the cytoplasm fragments into as many portions as there are nuclei and the parasite in this stage is known as the ‘‘segmented’’ or ‘‘mature’’ **schizont.** The uninucleate progeny are known as ‘‘**merozoites.**’’

The erythrocyte ruptures, releasing the merozoites, and accumulated pigment, into the plasma of the blood. The individual merozoites usually enter other erythrocytes. The terms ‘‘schizont’’ and ‘‘trophozoite’’ are not mutually exclusive. A **trophozoite** is the actively growing asexual stage regardless of its nuclear condition. As stated before, after Schaudinn’s work, published in 1903, for twenty years there was a generally accepted belief that schizogony was exclusively erythocytic. It is obvious from the work of James that intermediate stages exist between sporozoites and erythrocytic stages. Warren and Coggeshall in 1937 proved that during the ‘‘negative’’ phase of the blood, *P. cathemerium* parasites actually existed in the spleen, liver, bone marrow, and the site at which the sporozoites had been inoculated. These results were confirmed and extended to other species.

In the stages of the avian malarial parasites, two types have been recognized as exoerythrocytic, the *P. circumflexum* and *P. elongatum.*

Different terms are now used for the two categories: **exoerythrocytic stage** and the **pre-erythrocytic stage,** which comprise all forms of the first line of development between sporozoites and the first erythrocytic trophozoites. This includes the first generation **cryptozoites,** stemming directly from the sporozoites, and **metacryptozoites,** which follow the cryptozoites. The term ‘‘exoerythrocytic’’ cell means the stages in the cells other than the erythrocytes which are seen in blood-induced infections, and late enough in sporozoite-induced infections so as not to be thought of as pre-erythrocytic stages. Huff and Coulston have proposed the term ‘‘phanereozoites,’’ or ‘‘phanerozoic’’ stages for this category.

PLATE L.—CYCLE OF DEVELOPMENT OF MALARIAL PARASITES IN MAN AND MOSQUITO

1. Bite of mosquito. Injecting sporozoite into man from its salivary gland.
2-14, 2A-15A, 2B-12B. Blood stream of man.
15. Bite of mosquito. Receiving sexual forms from man.
16-24. Stomach of mosquito. Oöcysts formed on the outer wall of the mosquito's stomach.
25. Salivary gland of mosquito.
2, 2A, 2B. Free sporozoites.
3, 3A, 3B. Penetration of sporozoite into a parenchyma cell of the liver.
4, 4A, 4B. Beginning schizogony. Ring form.
2-9, 4A-10A, 4B-7B. Schizogony. Cause of fever.
5, 5A, 5B. Half-grown parasite.
6, 6A. Mature parasite.
7, 7A, 8A. Beginning division.
8, 9A, 6B. Division forms.
9, 10A, 7B. Merozoites.
10, 11A, 8B. Young male.
11, 12A, 9B. Adult male.
12, 13A, 10B. Young female.
13, 14A, 11B. Adult female.
15. Bite of mosquito. Inoculation of mosquito from patient.
16. Macrogametocyte.
17. Microgametocyte.
18. Fertilization.
19. Zygote.
20. Oökinete entering stomach wall.
21. Young cyst on outer stomach wall.
22. Cyst with sporoblasts.
23. Cyst with sporozoites.
24. Wandering sporozoites.
25. Sporozoites in salivary gland of mosquito.

CYCLE OF DEVELOPMENT OF MALARIAL PARASITES IN MAN AND MOSQUITO

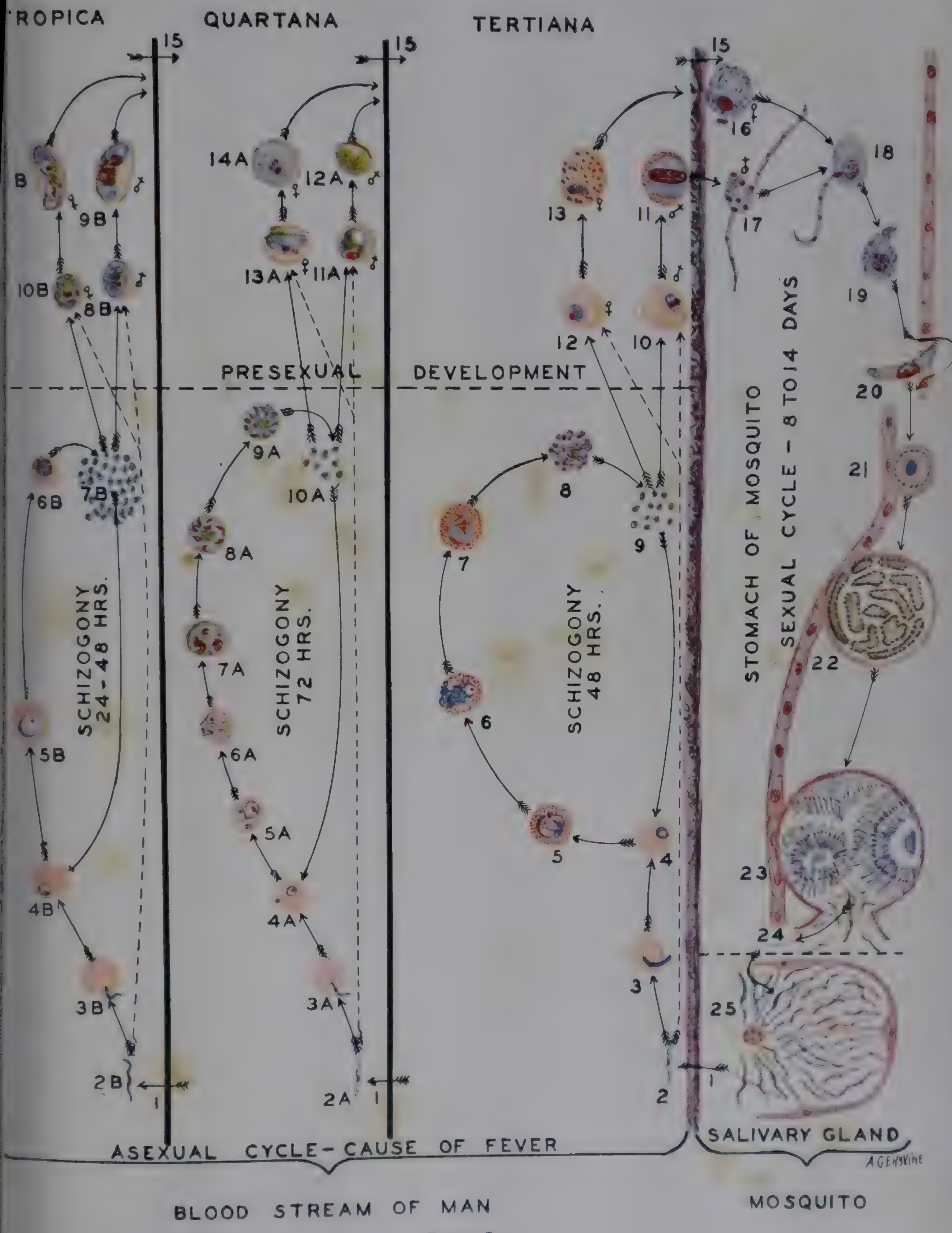
ROPICA
QUARTANA
TERTIANA
15
15
15
B
9B
10B
8B
14A
12A
13A
11A
13
11
12
10
16
17
18
19
20
21
22
23
24
25
PRESEXUAL DEVELOPMENT
6B
7B
9A
10A
8A
7A
6A
5A
4A
3A
2A
1
8
9
7
6
5
4
3
2
1
5B
4B
3B
2B
1
SCHIZOGONY 24-48 HRS.
SCHIZOGONY 72 HRS.
SCHIZOGONY 48 HRS.
STOMACH OF MOSQUITO
SEXUAL CYCLE - 8 TO 14 DAYS
ASEXUAL CYCLE - CAUSE OF FEVER
SALIVARY GLAND
A GERSKINE
BLOOD STREAM OF MAN
MOSQUITO

PLATE L.

Summary.—Sporozoites of *P. vivax* and *P. falciparum*, and presumably those of the other parasites, leave the blood after a few hours and undergo a pre-erythrocytic phase in the parenchyma cells of the liver. The ripe forms produce a group of young sporelike formed merozoites which seek blood corpuscles for further cyclical development. It must be remembered that there are two forms of development: one asexual, which is called "schizogony"; and the other, the sexual form, which develops gametes, called "sporogony."

The mosquito picks up the malarial parasite by biting a malarial subject. The male and female elements combine and produce their offspring in the stomach of the mosquito. In other words, the adult males, or microgametocytes, fertilize the adult females, or macrogametocytes. This occurs from twenty minutes to two hours after the bite. From this fertilization, the oökinete is formed. This pierces the wall of the stomach of the mosquito and starts an oöcyst under the outer epithelial area of the stomach. This occurs in about forty-eight hours. The oöcysts develop slowly, forming a large sporoblast with many thousands of sporozoites in the form of a large cystic mass on the wall of the stomach. Rupture of this cystic mass permits the sporozoites to enter the salivary gland of the mosquito. The entire development, provided the proper temperature prevails, takes place in from eight to ten days. By proper temperature is meant between 24 and 30° C. Sharp cooling hinders this development. From the salivary gland, the free sporozoite is inoculated into the next human subject and the next cycle of development in man begins.

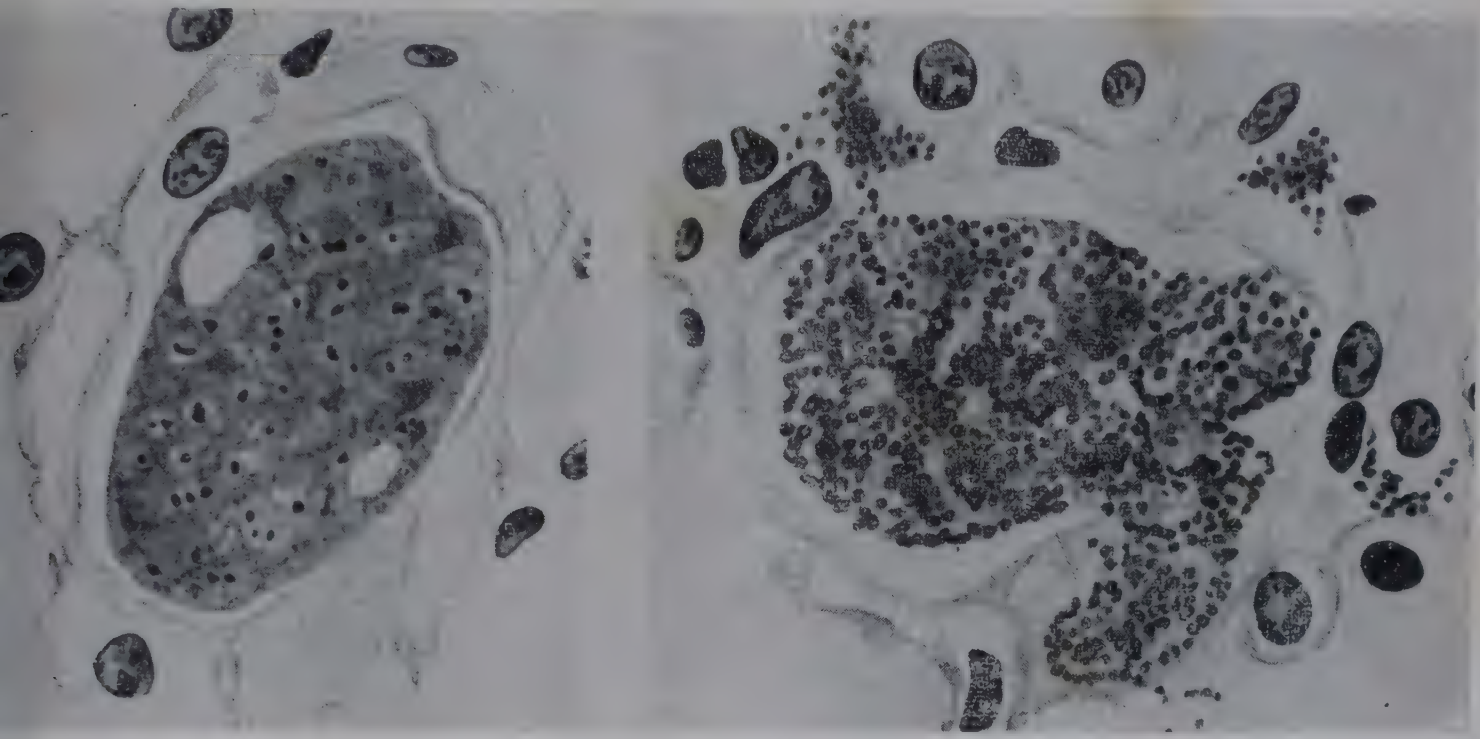

Fig. 480.—Pre-erythrocytic phase. Schizonts of *Plasmodium vivax* showing development in the human liver on the seventh day after experimental sporozoite infection. (After Shortt and Garnham: Tr. Roy. Soc. Trop. Med. & Hyg. **41**: No. 6, 1948.)

1. Plasmodium Vivax (Grassi and Feletti 1890) (Malaria Tertiana)

In the Giemsa-stained specimen, the youngest asexual form appears as a blue ring with a small red dot. The free interpretation of the ring is, according to Schaudinn, that it is a nutritional vacuole. In the youngest forms of the parasite may be seen distinct pigment (melanin) as fine granular brown or black irregular masses. The asexual forms may reach the full size of the red blood corpuscle. It is in this form of malaria, therefore, that the corpuscle seems to be increased in size by the presence of the parasite. The nucleus of this parasite divides into 12 to 22 very small nuclei, while the pigment is distributed usually into two large clumps. After a parasite reaches the stage of division, there is a destruction of the blood cells and a new cycle of development occurs. In the case of tertian malaria this takes about forty-eight hours and is responsible for the absence of fever during this time. The name ''tertian'' is used because the fever occurs every third day. If, however, there are two parasitic varieties in the same blood at the same time, development takes place on the third day, but every day or so paroxysms may occur, which is called ''tertiana duplicata,'' also known clinically as ''tertian fever.''

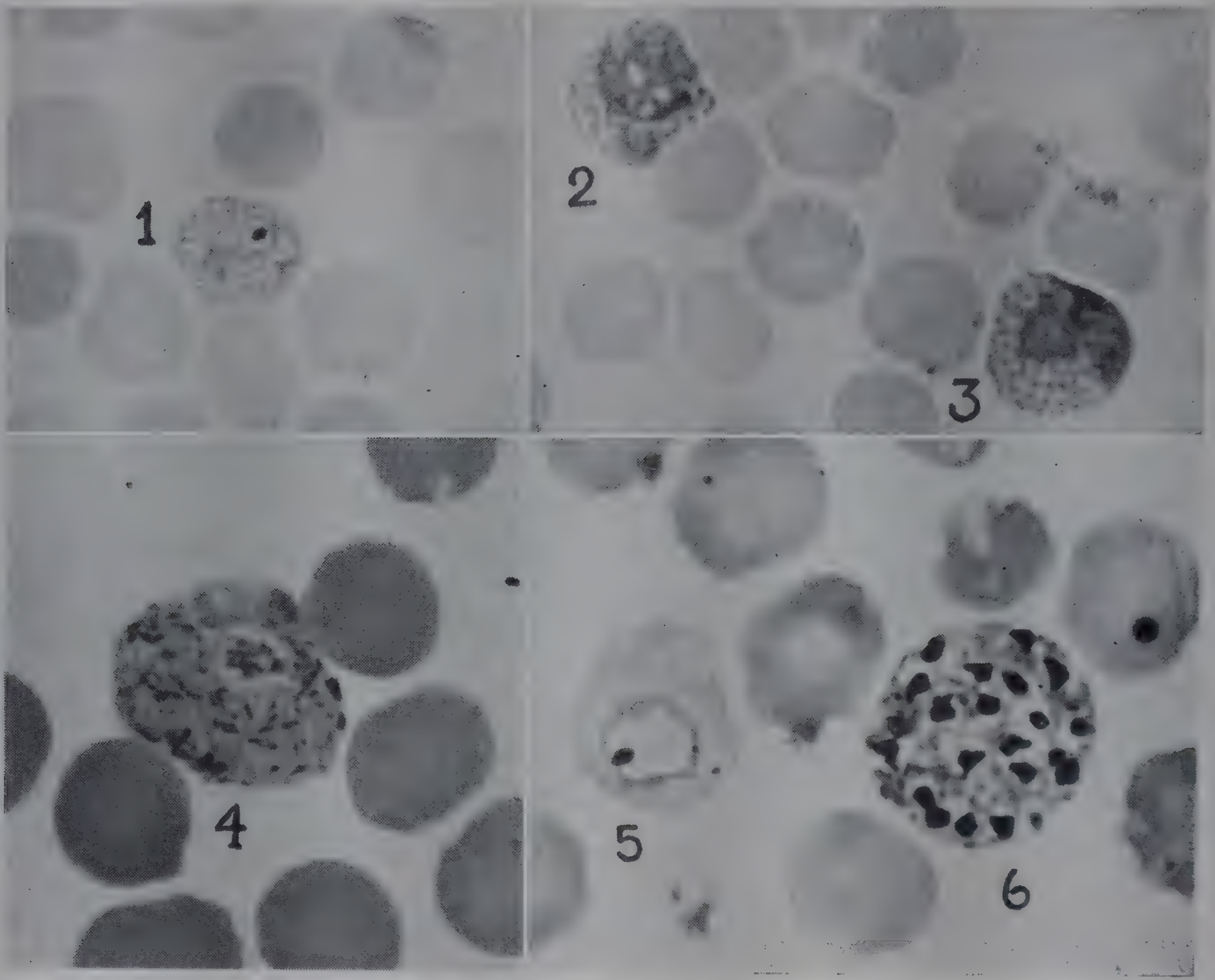

Fig. 481.—*Plasmodium vivax.* Film of peripheral blood stained with Giemsa. *1,* Young schizont, ameboid form, showing Schüffner punctation; *2* and *3,* two schizonts, ameboid form, in a further stage of development, each parasitizing one erythrocyte with Schüffner punctation; *4,* spherical gametocyte occupying the entire enlarged erythrocyte; *5,* young trophozoite, ring form, parasitizing a pale, enlarged erythrocyte; *6,* completed schizogonous division, numerous merozoites, which fill the entire enlarged erythrocyte. Compare the size of the parasitized erythrocytes with those without parasites. (Original photomicrograph of Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

The sexual forms, microgametocytes and macrogametocytes, in their youngest stage, form round or oval blue bodies with chromatin granules in the center or on the side. They retain their round form for a long time, but they do not show any ameboid movement. In the older stages, the cytoplasmic-rich female gametes are stained intensive blue. The chromatin-rich nucleus is at one side and often shows a light aureola. They can attain twice the size of the blood corpuscle. The male sexual forms show the same figure, but remain small. They are poor in cytoplasm and therefore show more nuclear substance. They also stain a lighter blue and often are reddish. The nucleus is lobulated and shows red chromatin lines. Fully developed ripe male forms show a rich fine granular brown black pigment which is distributed all over the parasite. In the unstained specimen this shows active Brownian molecular movement. The sexual forms can be distinguished from the asexual parasites by this pigment in the fresh specimen as well as in the thick drop preparation. We have observed double infection of asexual and sexual forms in the same specimen of blood.

The asexual forms show clumping of the pigment; the sexual forms show no clumping but a generally scattered pigmentation.

The red blood cells in tertian infection are very pale and are increased in size by virtue of the presence of the parasite. They show in the Giemsa stain very fine, later coarser, so-called Schüffner stippling. Alkalinizing the stain makes the Schüffner stippling more distinct, whereas neutralizing the water makes the parasite stand out more distinctly. The schizogony or asexual division of *P. vivax* shows 12 to 22 nuclei, while that of *P. malariae* shows no more than 12. This is a very important point of differentiation.

The rings of *P. falciparum* are small and represent about one-fifth of the diameter of the cell, whereas the rings of *P. vivax* are large, representing one-third of the diameter of the cell. The rings of *P. falciparum* also show two or more nuclei, while those of *P. vivax* show but one, rarely two. Also it must be remembered that the rings of *P. malariae* are larger, but the red blood cell itself is not enlarged.

2. Plasmodium Malariae (Grassi and Feletti 1890) (Malaria Quartana)

The quartan parasite resembles the tertian parasite but it is smaller and not as actively motile. The youngest asexual forms are rings. Older forms always appear as bands. Dependent upon the age, there are either small or wide band forms. The chromatin appears near the edge; there is an elongated nucleus. There is a very fine and light golden yellow pigment (melanin) close to the edge. A completely developed parasite has wide bands with divided nuclei. The divisions are between 8 and 12 but seldom up to 16, as is seen in *P. vivax*. These forms attain about the normal size of the red cell. The cycle of the development period for *P. malariae* is seventy-two hours. Several varieties, however, may exist in the same individual so that there is a double infection with daily fever or two days of fever in succession, the so-called "quartana duplicata" or "quartana triplicata." The sexual forms show the same form and staining as the *P. vivax* but are never larger than a normal red cell. The red cells in quartan malaria are not enlarged, not blown up, and not stippled, but are more globular. In quartan malaria, the parasites are rather scarce in peripheral blood.

3. Plasmodium Falciparum (Welch 1897) (Malaria Tropica) (Estivo-Autumnal Malaria)

The youngest asexual forms are very small rings. Older stages show more protoplasm and appear as large rings, the so-called "cycle" ring forms. They usually show two chromatin granules which lie close together. The ripe asexual forms, especially in comatose cases, are found in the peripheral blood.

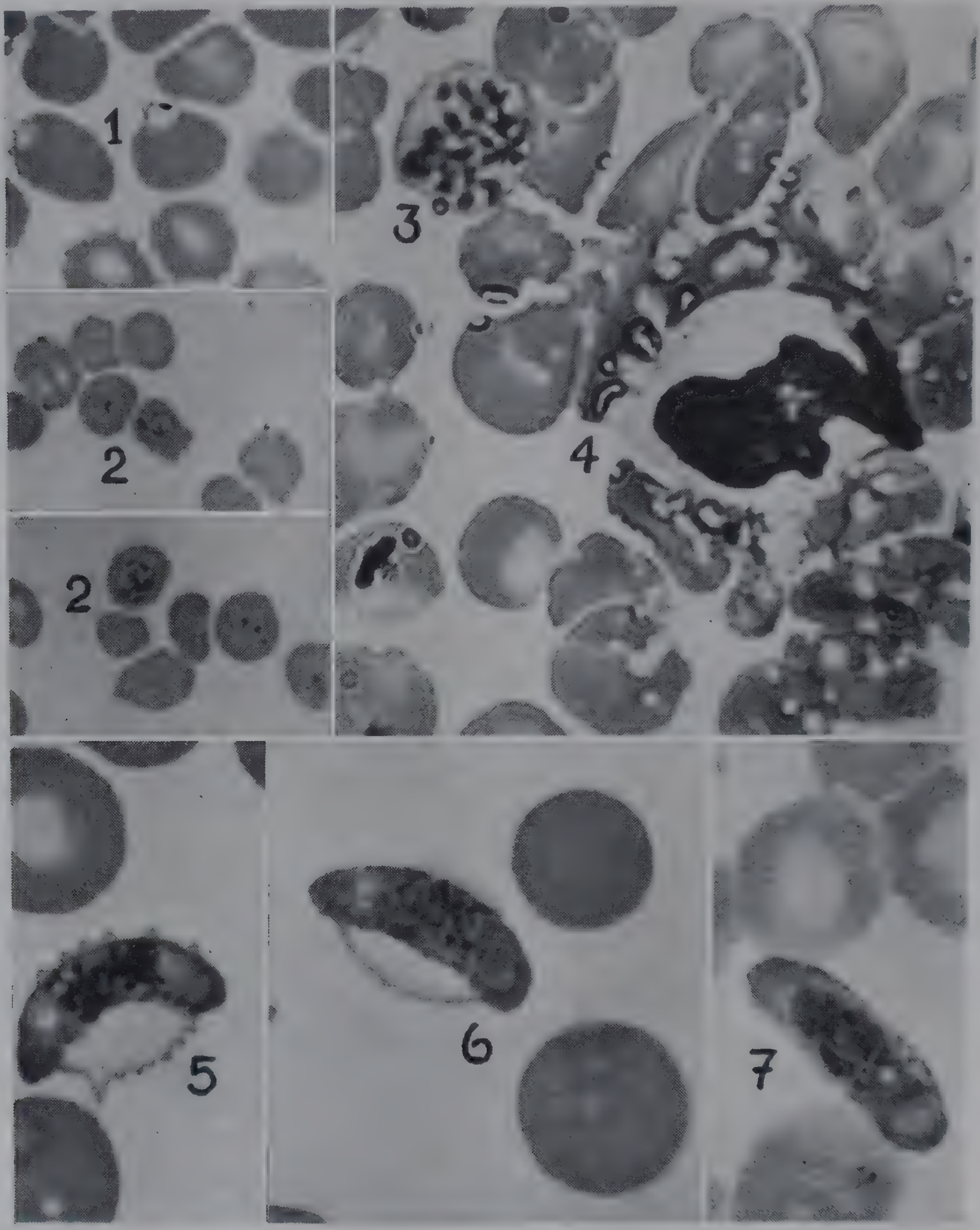

Fig. 482.—*Plasmodium falciparum.* Blood film stained with Giemsa stain. *1,* Young trophozoite, ring form; *2,* fully developed schizont; *3,* schizont divided into numerous merozoites, rosette form; *4,* an endothelial cell in peripheral blood; *5, 6,* and *7,* typical crescent form, banana, bean, or kindey shaped, of the gametocyte. Note in *5* the arch of the denticulated border which encloses the cavity of the gametocyte, as well as the festooned edges, representing the remnants of the parasitzed red cell; and in *6,* note the smooth edge of the arch which incloses the cavity of the gametocyte, representing the rest of the parasitized red cell. (Original photomicrographs of Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

The dividing form shows 8 to 12 to 32 nuclei. Development takes forty-eight hours. Sexual forms of the tropical parasite are roundish, often long oval. The riper forms are long, light, concave-convex pictures called the "crescent" form. The female forms, with Giemsa, stain an intensive blue. They lie squarely in the middle of the red blood cell or near one peripheral border. The male forms are lighter, often reddish, with a lobulated nucleus with pigment.

The red blood cells in this form with asexual parasites present are not enlarged. They show a deep red spotting in the typical chromatin stain, the so-called "Maurer or Christopher pernicious spotting." They show irregular spots.

Plasmodium Ovale (Stephens 1942)

P. ovale has sufficient morphologic characteristics to differentiate it from the *P. vivax* and from the *P. malariae*. It is round or oval with exceptionally ameboid schizonts, and fewer merozoites than the vivax, 8, which compose the rosette form. These rosettes are very small by comparison with those of *P. malariae*. The gametocytes of *P. ovale* are spherical and smaller than those of the *P. vivax* and are found in the red cells in oval form, with slightly decolorized and indented borders, at times hypertrophic, and presenting heavy Schüffner punctation in red cells parasitized by the young trophozoites. At times the parasitized erythrocyte is weakly stained and seems almost free of Schüffner punctation. The *P. ovale* increases the size of the erythrocyte. The rosettes have few merozoites. It is in this particular that it resembles *P. malariae*, but it differs from that form by its oval shape and indented borders and by the abundant and frequent Schüffner punctation, observed so often in erythrocytes containing the *P. ovale* and not in those parasitized by *P. malariae*.

TABLE 183.—DIFFERENTIAL POINTS OF THREE TYPES OF MALARIAL PARASITES

	PLASMODIUM VIVAX	PLASMODIUM MALARIAE	PLASMODIUM FALCIPARUM
Type of Malaria	Tertian	Quartan	Tropical (Estivo-Autumnal), Falciparum
Effect on Size of Red Blood Cell	Enlarged	Reduced	No change
Effect on Color of Red Cells	Pale	Intensified	No change
Stippling of Red Blood Cell	Schueffner punctation may be observed	None	Pernicious maculation may be seen
Size of Ring	Large (⅓ diameter of red blood cell)	Medium or small	Very small (⅕ diameter of red blood cell)
Number of Nuclei in Each Ring	1, rarely 2	1, sometimes 2	2 or more
Number of Nuclei in Division Forms	12-22	Never more than 12	8-12-32
Shape of Asexual Forms	Very many different forms	Usually band	Not seen (ring)
Shape of Mature Forms	Oval or round	Oval or round	--
Chromatin of Mature Forms	At one side	Elongated forms near edge	--
Shape of Sexual Forms	Round or oval	Round or oval	Crescent
Size of Sexual Forms	Usually slightly larger than red blood cell	Smaller than red blood cell	Diameter: about ½ red blood cell; length: slightly longer than red blood cell
Pigment of Sexual Forms	Scattered	Clumped	Centralized
Cycle of Asexual Development	48 hr.	72 hr.	48 hr.
Yellow Pigment in Asexual Forms	None	Always present	Occasional

Irregular, Relapsing, and Recurring Malaria

Irregular, relapsing, and recurring malaria are rather frequent, especially in treated patients. The parasites may be difficult to find in the peripheral blood stream. Sternal puncture can be helpful in such cases.

Diagnosis of Malaria by Laboratory Methods

Methods of Examination.—

The parasites may be recognized in *unstained preparations* by observing small brown or black granules within them. They show a distinct dancing motion. The unstained specimen is made by simply placing a drop of blood between a cover glass and slide.[1]

The fully developed parasite can be well seen in the stained specimen, the Giemsa, the Romanowsky, the Giemsa-Romanowsky stains combined, or Wright stain. With these stains, the protoplasm is stained blue and the chromatin of the nucleus red. (See Plates XLIX and L.)

1. Take a drop of blood from the ear with the usual precautions, place it on a clean slide, superimpose a cover glass, ring with petroleum jelly, and examine at once with a high dry lens and then with the oil immersion lens.
2. Stain margin-free films with Giemsa or Wright stain.
3. Stain thick drop specimens in the usual way.
4. Use the Barber-Komp method.

This diagnosis can best be made by finding the plasmodia in direct blood examinations, thin or thick films, or by thick drop. For the thick drop method for the diagnosis of malaria use the method of Barber and Komp. Staining with Giemsa, or Wright and Giemsa combined, affords an admirable method of diagnosis. Most cases of symptomatic malaria without treatment will show plasmodia. Even if the infection is latent, thick films or thick drops will reveal them. If this fails, then resort must be had to methods of driving the plasmodia from their hiding places in the body out into the peripheral blood stream. For this purpose, the subcutaneous injection of 1 mg. of Adrenalin serves quite well. At the same time one may expose the splenic area to x-rays, followed by physical exercise. Plasmodia usually appear in the blood shortly after the administration of Adrenalin. Others use strychnine nitrate, 2 mg., subcutaneously administered, for the same purpose.

Concentration Methods.—Some years ago, the author had an opportunity to witness Bass and Johns' excellent method of concentration of malarial plasmodia in their laboratories in New Orleans.

Their method advises 5 to 10 c.c. of blood withdrawal from an arm vein. Receive it into a syringe containing a small amount of sodium citrate solution. Eject the blood into a 50 c.c. centrifuge tube and rotate at 2,500 r.p.m. Pipette off the upper column of red cells and transfer to a smaller test tube, centrifugalize at the same rate of speed until the cells are deposited at the bottom of the tube. Make thin film preparations by means of a pipette which takes off a small amount of this sediment, place it on glass slide, place another slide on top and push it along to make a film that is rather thin. Dry and stain at once.

Malarial Forms Found in Blood Films

In cases of single infection with *P. vivax* or *P. malariae*, one will find, in the blood films of such patients, schizonts which practically fill the structure of

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[1] For technic of staining, refer to pages 615 ff. and 650 ff.

the red blood cells; mature and segmenting schizonts; free merozoites, and gametocytes. In the case of infection with *P. falciparum*, only ring forms are found in the blood at the time of the paroxysm, but if the infection has existed several days, crescents will be found. Shortly after paroxysms of *P. vivax* and *P. malariae* infections, ring forms of trophozoites and young gametocytes are found together with young pigmented forms. The day following the paroxysms in infections with *P. vivax,* we find young gametocytes. In the case of *P. malariae,* the next day one finds partly developed schizonts and gametocytes. With *P. falciparum,* only ringlike trophozoites and gametocytes are found at the end of 24 hours. When 36 hours have elapsed, in *P. vivax* infections, pre-segmenting schizonts, ring forms and gametocytes are found. At such a time *P. malariae* cases show half grown and fully grown schizonts and gametocytes. With *P. falciparum,* there are ring forms of gametocytes. In double infections there will be seen the same forms already described plus many half grown schizonts.

HUMAN HELMINTHOLOGY[1]—NEMATHELMINTHES

The animal kingdom is divided into Protozoa and Metazoa. Metazoa are further divided as follows:

Metazoa	Vertebrates	Bilaterally symmetrical	Artiozoa
	Invertebrates	Bilaterally symmetrical	
		Radically symmetrical	Phytozoa

The Artiozoa, which comprise all those animals bilaterally symmetrical, are divided into two large groups: vertebrates and invertebrates. Of the invertebrates, the Helminths and the Arthropods are of interest here.

Helminthology, as the word indicates, is the study of worm parasites, or helminths. Helminths include the roundworms, the flatworms, and the tapeworms.

The Helminths are divided into two phyla of importance: phylum Nemathelminthes and phylum Platyhelminthes. The first phylum is divided into three classes: Nematoda, Acanthocephala, and Gordiacea; the second into five classes, of which only two are of interest to us: Cestoidea and Trematoda. The following diagram gives the characteristics of each:

		Phyla		**Classes**
Helminths or Worms	Round body, chitinous integument, coelomic cavity present, usually dioecious	Nemathelminthes	Usually with complete digestive tract	Nematoda
			Posterior part of the digestive tract atrophied in the adult	Gordiacea
			Digestive tract absent	Acanthocephala
	Flat body, soft integument, without coelomic cavity, usually hermaphrodites	Platyhelminthes	Digestive tract absent; segmented body	Cestoidea
			Incomplete digestive tract, without anus	Trematoda

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[1] In the preparation of the preceding edition we wish to acknowledge with appreciation the excellent translation work by one of our assistants, Mr. Rubin Marshosky, of Panama City, R.P.

Although this classification of the Helminths does not exactly follow the Zoological Classification, we have adopted it for its simplicity and practical value in clinical work.

PHYLUM NEMATHELMINTHES

The most important Nemathelminthes which habitually parasitize man belong to the class Nematoda.

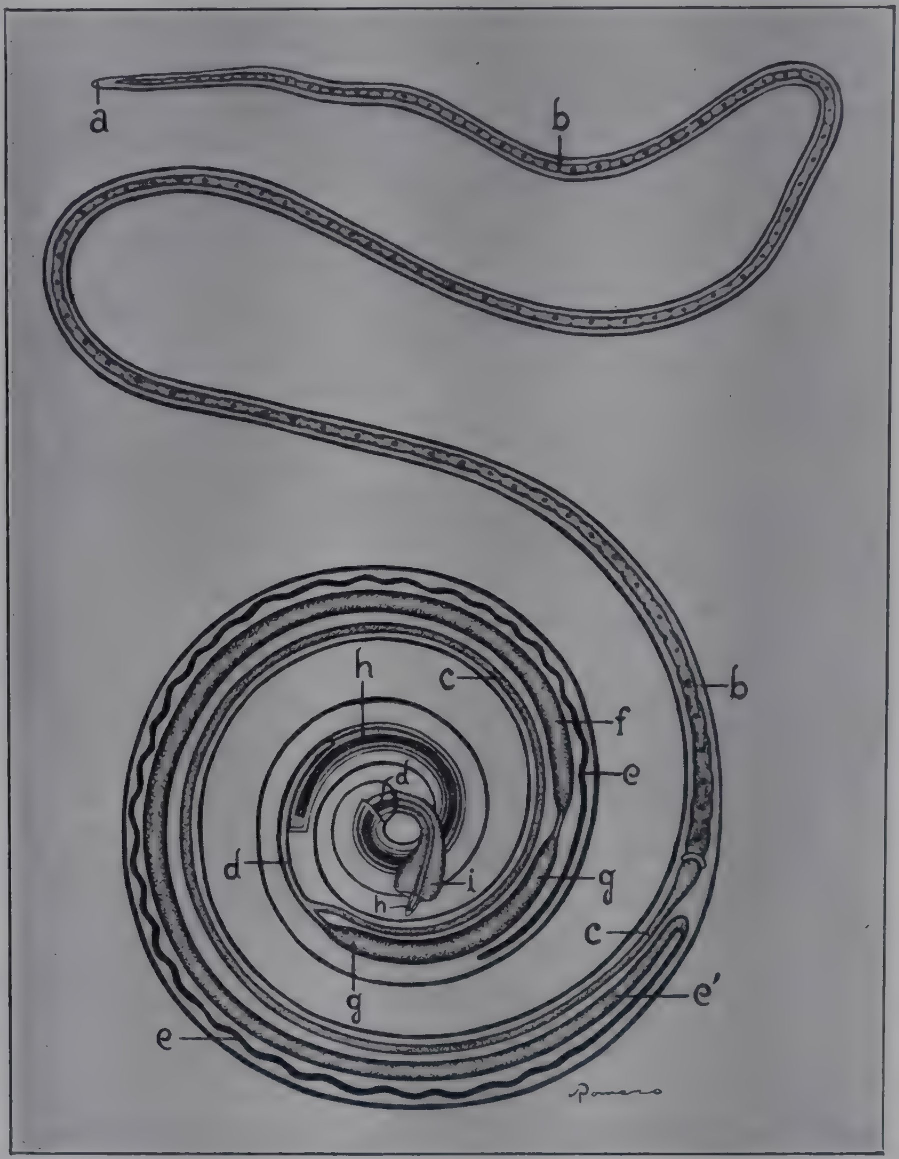

Fig. 483.—Male *Trichuris trichiura*. *a, b, c, d,* Digestive tract; *a,* mouth in the anterior end of the parasite; *b, b,* esophagus occupying the entire length of the thin portion of the parasite and formed by a fine duct which passes through a single layer of cells; *c, c,* intestine; *d, d,* cloaca; *e, e′, f, g, h, i,* male genitalia; *e, e,* testicle; *e′* vas deferens; *f,* seminal vesicle; *g, g,* ejaculatory duct; *h, h,* spicule; *i,* sheath or thorny prepuce. (After Guiart, modified by Kourí: Lecciones de Parasitología y Medicina Tropical. Courtesy of Editorial Profilaxis S.A., Havana, Cuba.)

Class **Nematoda**

Nematodes are roundworms, vermiform, elongated, and cylindrical, whitish or pink, at times with dark bands.

Order **TRICHURATA**

TRICHURIASIS

Family **Trichuridae** Railliet 1915

The type species of this family is *Trichuris trichiura*, a parasite of man.

Trichuris Trichiura (Linnaeus 1771)

Trichuris trichiura, the whipworm, is usually found clinging to the intestinal mucosa, with the thin anterior portion buried in the chorium beneath the epithelium. It lies superficially parallel to the mucosa. It is a hematophagus parasite.

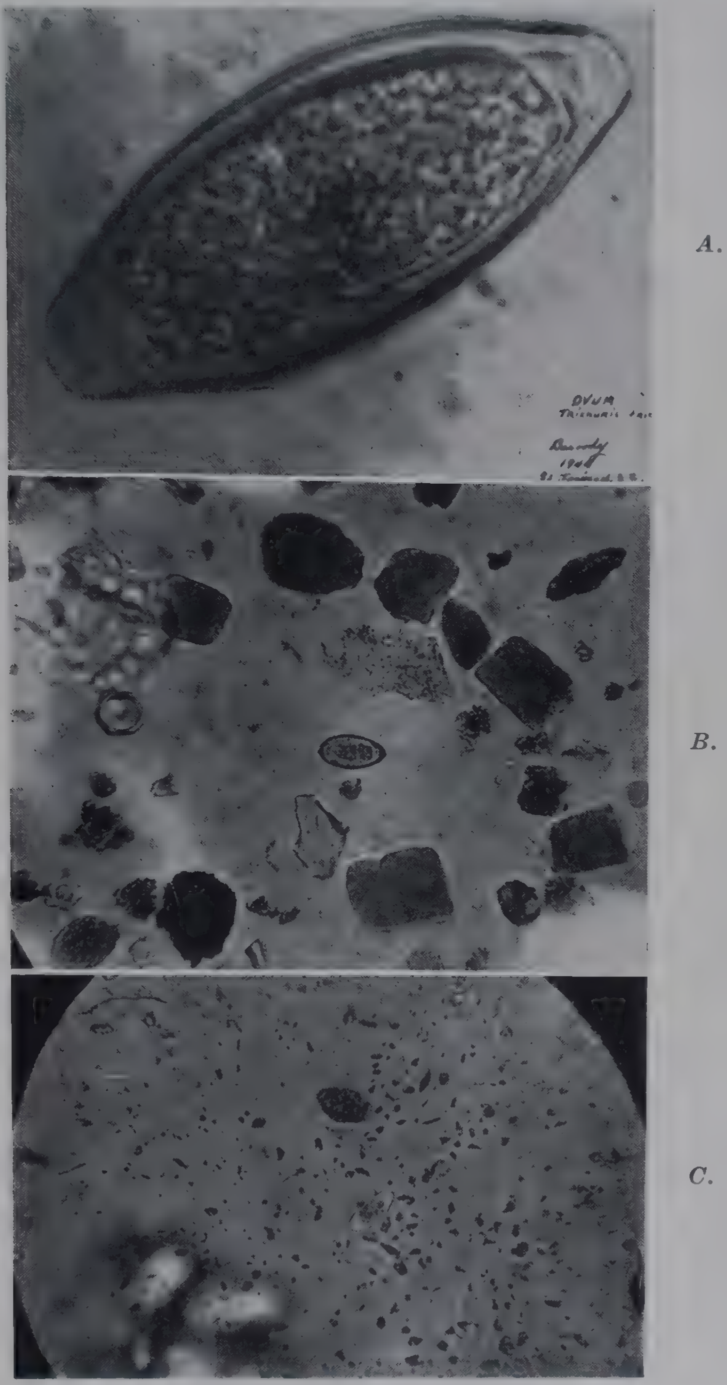

Fig. 484.—*A*, Egg of *Trichuris trichiura* (greatly enlarged). *B*, Egg of *Clonorchis sinensis*. *C*, Cyst of *Isospora hominis*. Water centrifugal sedimentation concentration method of Baroody and Most. (Original photomicrographs of Dr. B. J. Baroody.)

Eggs.—Eggs are very characteristic. They are approximately 50 microns long by 25 microns wide, symmetrically narrowed at the poles. They are brown due to the presence of bile. The shell is thick and is perforated at the poles. Each orifice contains a refractive, hyaline, colorless albuminous plug.

Parasites.—The female is approximately 4 to 5 cm. long. The thick portion is approximately half as long as the thin portion and describes an arc, the concave side facing the ventral part of the body. The esophagus is a thin

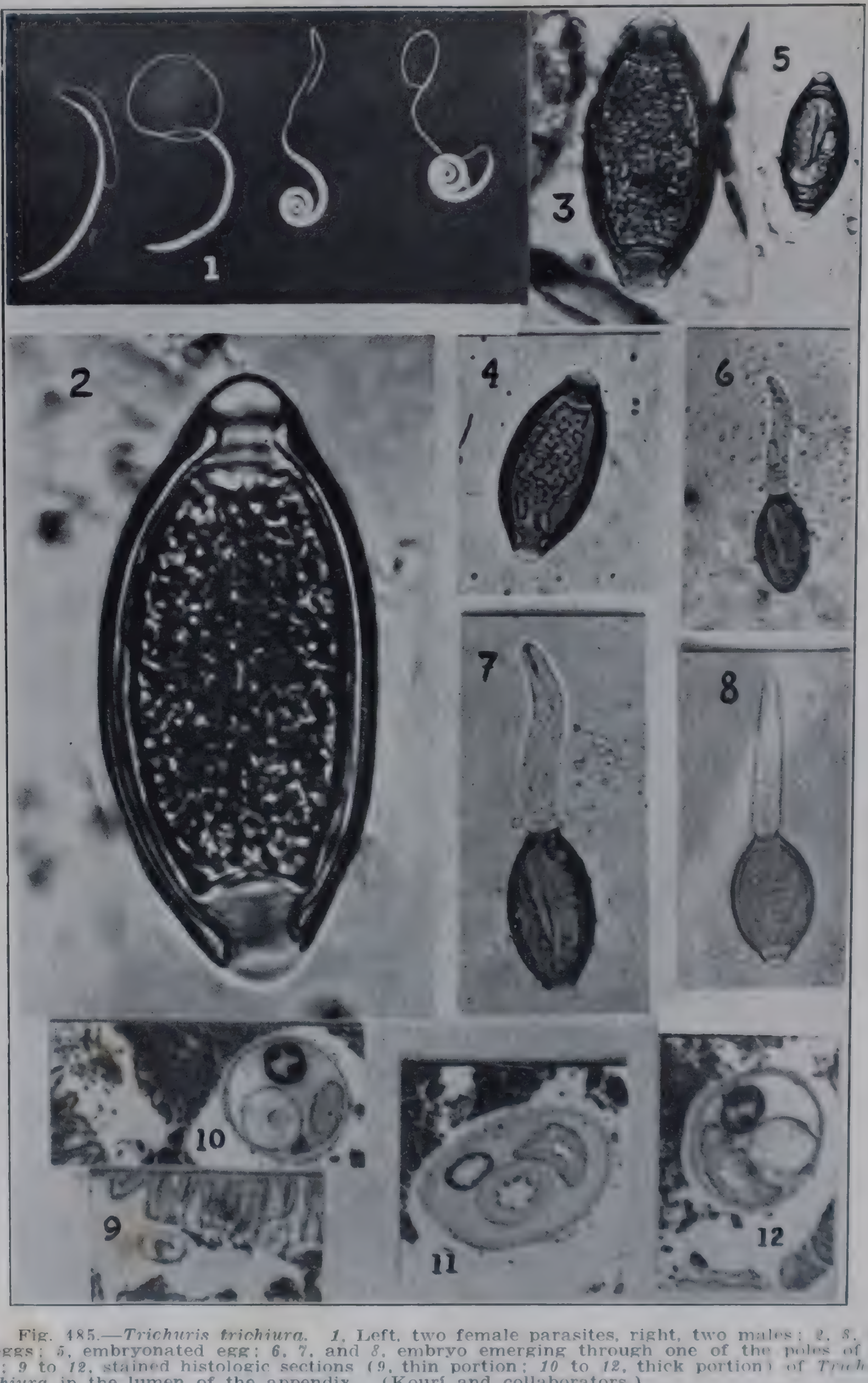

Fig. 485.—*Trichuris trichiura.* *1*, Left, two female parasites, right, two males; *2*, *3*, and *4*, eggs; *5*, embryonated egg; *6*, *7*, and *8*, embryo emerging through one of the poles of the egg; *9* to *12*, stained histologic sections (*9*, thin portion; *10* to *12*, thick portion) of *Trichuris trichiura* in the lumen of the appendix. (Kourí and collaborators.)

tube extending throughout the thick portion, ending in the anus. The genital organs lie in the thick portion. The uterus is broad and packed with eggs.

The male is smaller than the female, 3 to 4 cm. long with the thicker end spirally coiled, the convex surface lying ventrally. From the cloaca, in the thicker portion, extends a spicule, 2.5 mm. long, which bears a sheath or prepuce beset with spines.

The parasites are pink when alive, white in Formalin.

Life Cycle.—The life cycle is given in Fig. 486.

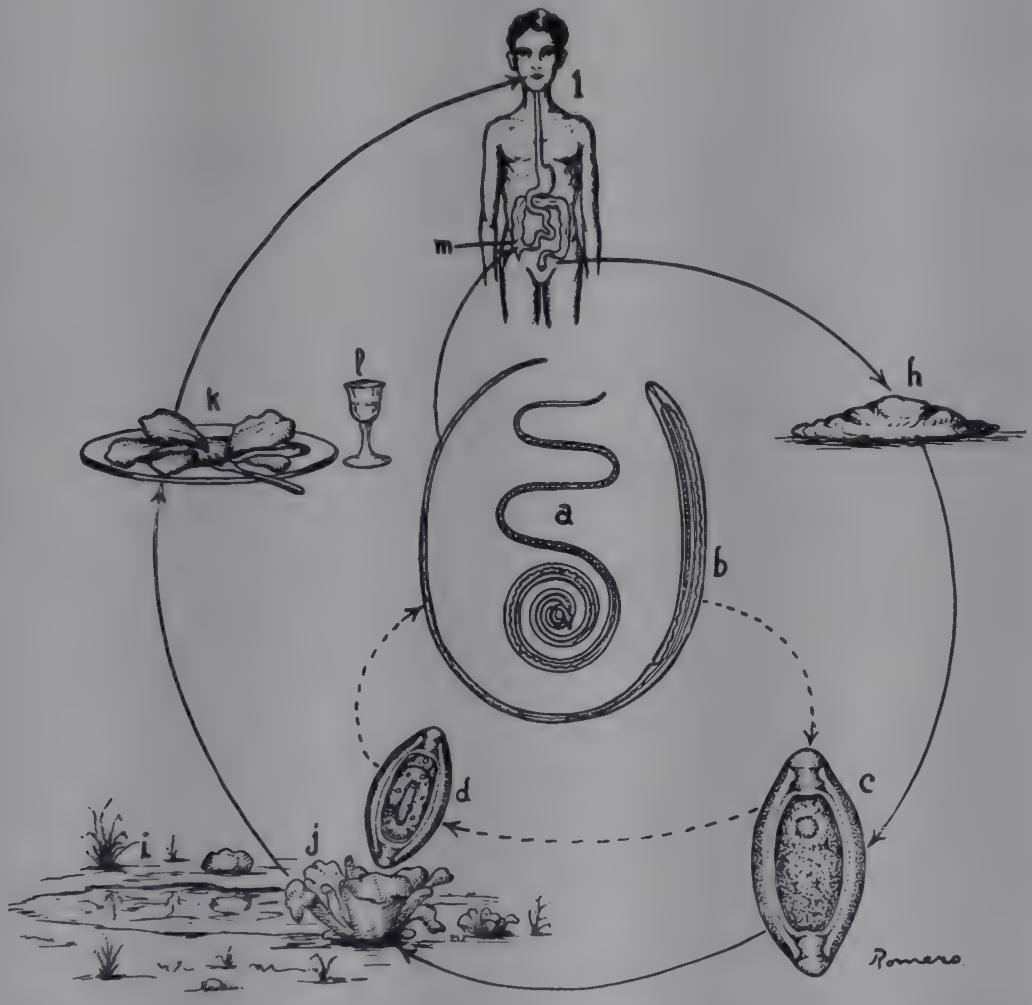

Fig. 486.—*Trichuris trichiura.* Life cycle. The male and female (*a* and *b*) localize primarily in the cecum of man (*1*). The female lays eggs (*c*) which are passed in the feces (*h*). Shortly after, they are embryonated (*d*), becoming the infective form. When ingested in contaminated food (*k*, *l*), the embryo is set free in the intestines and develops into the adult male (*a*) or female (*b*) parasite. After copulation, the female lays fertilized eggs (*c*) which are passed in the feces (*h*), beginning a new cycle. (Original drawing of Drs. Kourí and Basnuevo.)

Life Span.—The life span is unknown and is very difficult to calculate. Premunition is not believed to exist. For this reason, reinfestation is possible, and thus it is very difficult to calculate the life span, the more so because this parasite is cosmopolitan.

Epidemiology.—The infesting form is the embryonated egg, which is very resistant to adverse environmental conditions. This explains the frequency of parasitism since the opportunities of infestation are numerous in man. Eating vegetables containing embryonated eggs and putting the contaminated hands in contact with the mouth are two important modes of infestation.

Diagnosis.—Accurate diagnosis can be made only by thorough microscopic examination of the feces. The presence of the characteristic eggs is sufficient evidence.

In massive parasitism, presence of eggs is constant in fecal material, but in mild parasitism, negative phases exist, during which eggs are not found even with concentration methods.

In order to judge the efficacy of an antitrichuric drug it is necessary to submit it to parasitic control:

1. Count the eggs microscopically in the stools before treatment, taking an average of two or three serial examinations.
2. Count adult parasites expelled during treatment.
3. Count eggs microscopically after treatment and take the average of two or three serial examinations.

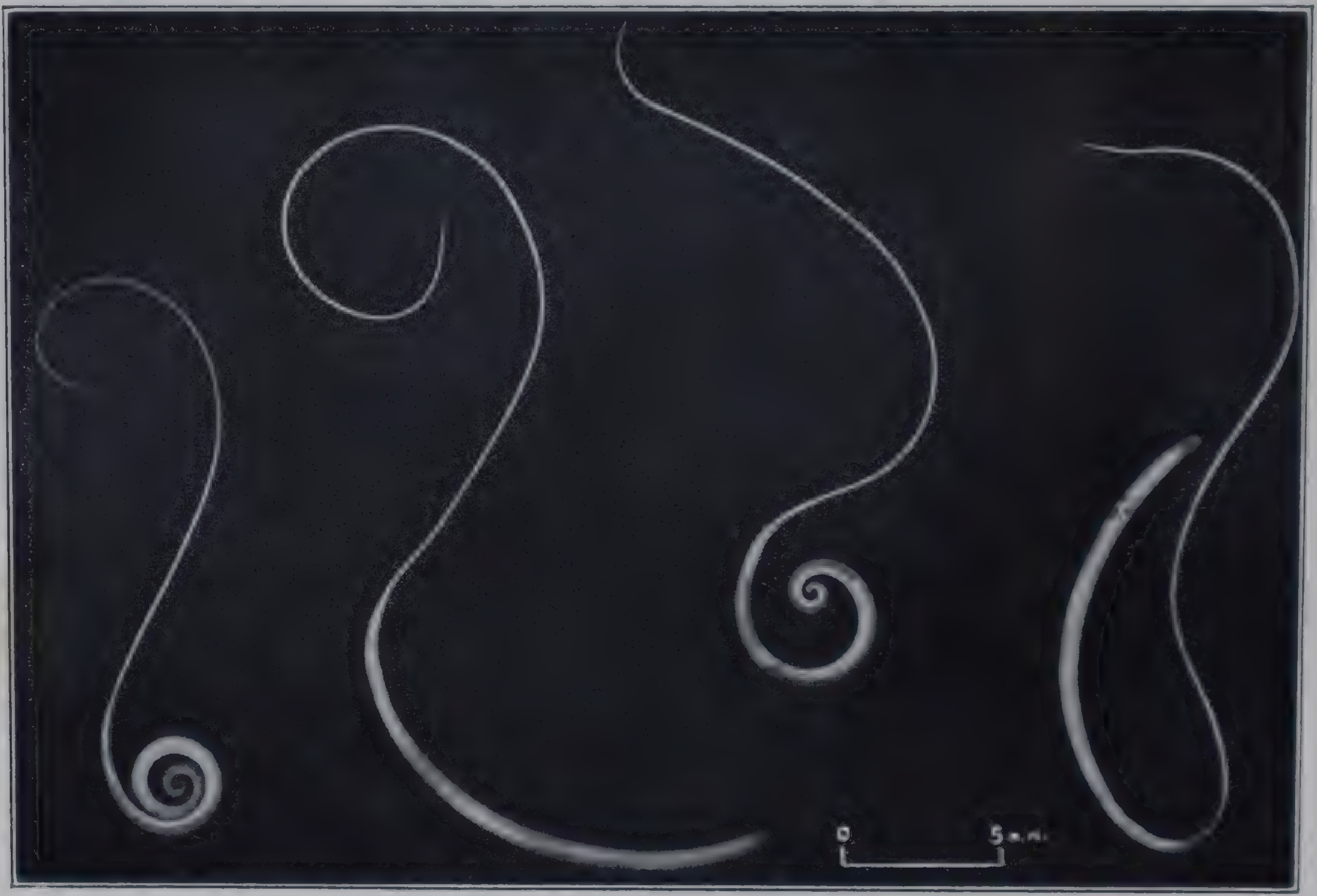

Fig. 487.—*Trichuris trichiura.* Photograph of male and female *Trichuris trichiura.* Millimeter scale. (Original photograph of Kourí.)

Absence of Trichocephalus eggs from the stools after treatment does not necessarily imply complete cure of the parasitism, since it is known that negative phases exist in mild infestations. Nor does the persistence of the eggs after treatment necessarily mean that the drug is ineffective, because if the eggs have not been counted one cannot ascertain whether or not the number of parasites has decreased, or how much the decrease has been. Counting the number of parasites expelled during treatment, however, gives an index of the efficacy of the drug used.

TRICHINOSIS

Trichinella Spiralis (Owen 1835) Railliet 1895

Geographical Distribution.—The parasite is cosmopolitan except in the Philippines, Puerto Rico, and Australia. Trichinosis is particularly prevalent where pork is eaten raw or insufficiently cooked; more frequent in the Northern Hemisphere than in the tropics. It is common in Europe and in the United States, but is relatively rare in Africa and South America. To date this parasite has not been observed in Cuba, either in humans or in animals.

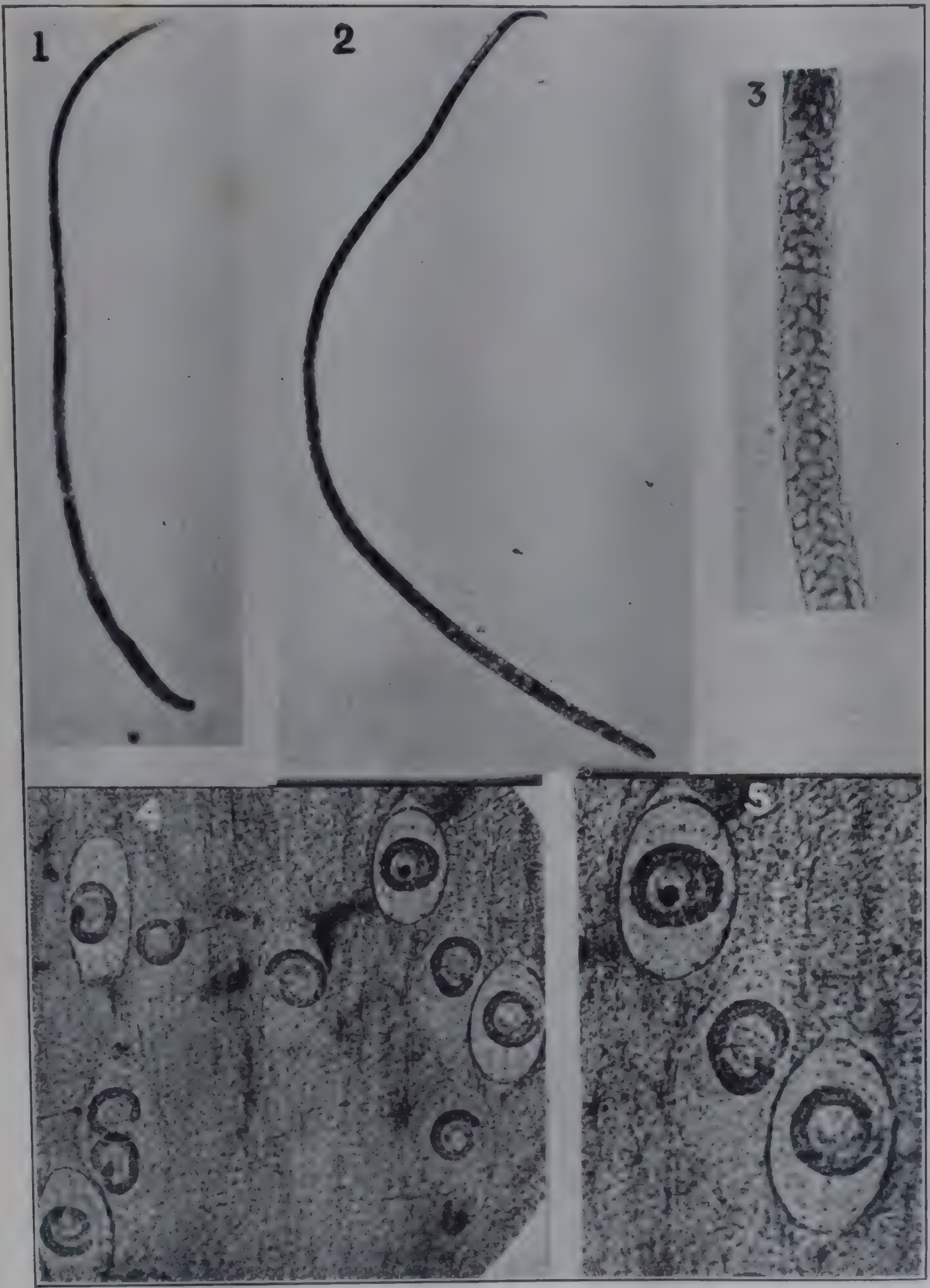

Fig. 488.—*Trichinella spiralis.* *1,* Male; *2,* female; *3,* magnified body of female. (Experimental infestation of swine. Material supplied by Dr. G. Bachman.) *4* and *5,* Larva in diaphragm of an experimentally infested white rat. (Originals of Kourí.)

Etiology.—The disease is caused by encystment of the larval forms in the striated muscles. The adult forms live in the small intestine.

Characteristics of the Parasite.—The male measures 1.4 to 1.6 mm. in length and 0.04 mm. in diameter. The anterior part of the body is narrowed; the orifice of the cloaca is terminal and lies between the two caudal appendages;

the two pairs of papillae are internal and dorsal, one behind the other. The cloaca is evertible for copulation. The female measures 3 to 4 mm. in length and 0.06 mm. in diameter; its anus is terminal.

This worm in its adult stage inhabits the small intestines of man, pig, wild boar, rat. The young do not leave the body of the host but become encysted in the muscles.

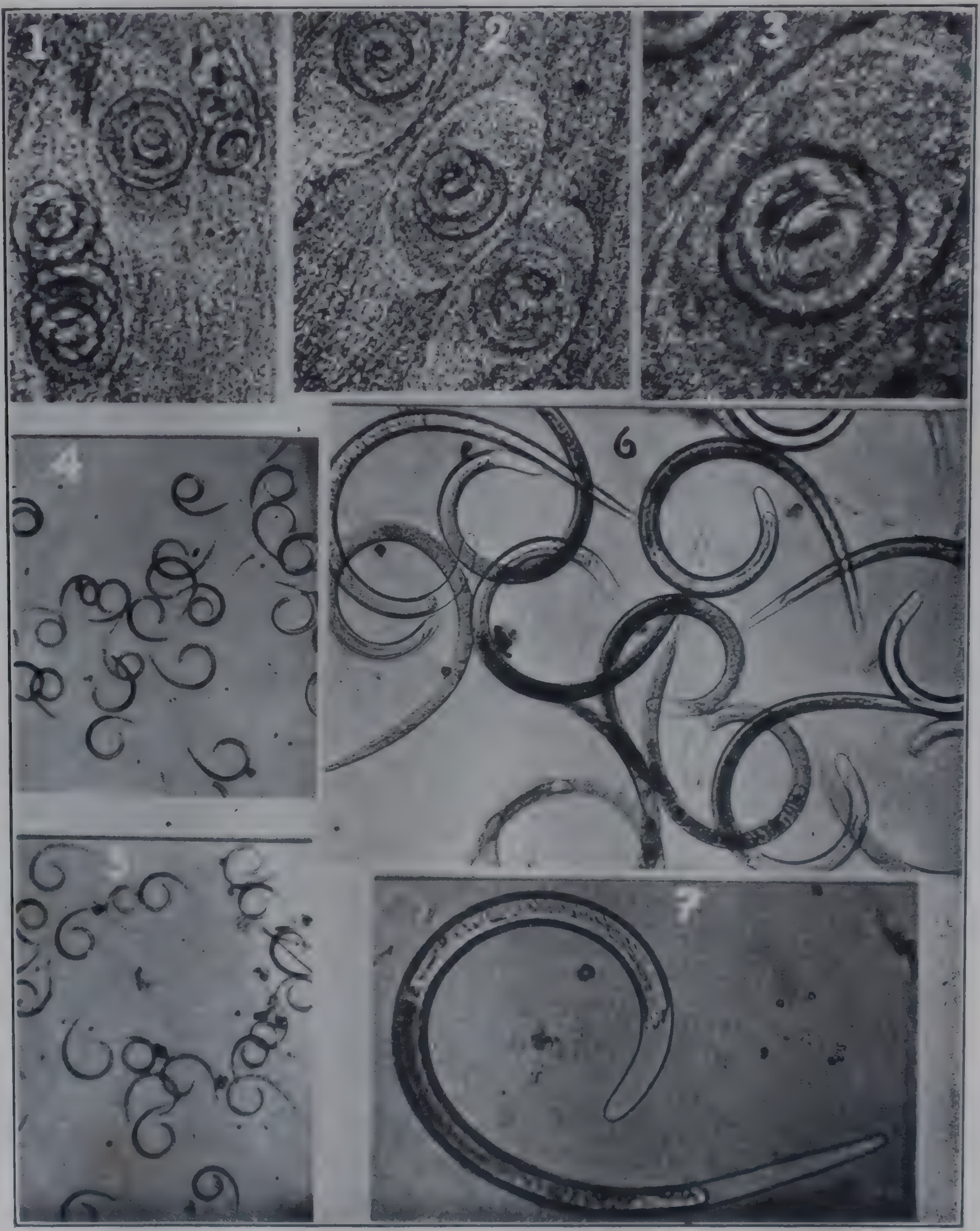

Fig. 489.—*Trichinella spiralis.* (Experimental infestation of white rat. Material supplied by Dr. G. Bachman, Puerto Rico.) *1* to *3*, Larvae in diaphragm muscle; *4* to *7*, larvae freed by artificial digestion of muscle. Various magnifications. (Original of Kouri.)

Development of *Trichinella spiralis* takes place in the intestines of experimental animals. The encysted trichinellae escape from their capsules, which are destroyed by the gastric juices. They then enter the duodenum and jejunum, where they mature. Soon after copulation, the males die and the females bore more or less deeply into the villi or into the mucous membrane and thus reach the lymph spaces. Some pierce the intestinal wall and are found in the mesentery and glands. The eggs are deposited in the lymph spaces. The larvae are carried along by the lymph stream to the heart. The young brood is distributed from the heart throughout the entire body, but the conditions necessary to their further development are found only in striated muscles; the young nematodes penetrate the capillaries, reach the intramuscular connective tissue, and then enter the fibers. On the ninth or tenth day after infestation the first worms have reached their destination; but further invasions are constantly taking place because the intestinal worms live from five to seven weeks, and continue to produce their young. Trichinae in their encysted condition may remain alive and capable of development for years.

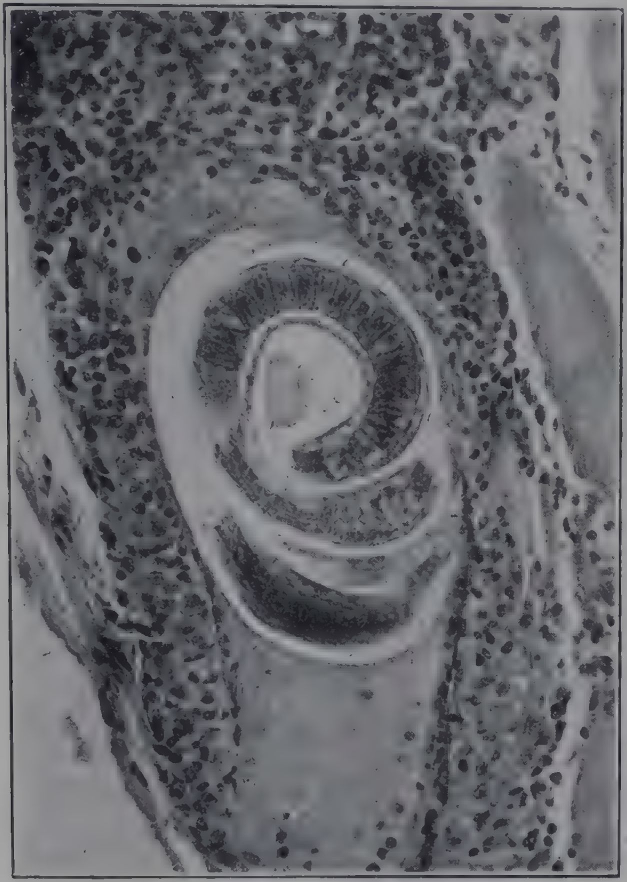

Fig. 490.—Section of *Trichinella spiralis* in muscle.

Symptomatology.—Infested patients have symptoms of gastrointestinal disturbance, nausea, vomiting, etc. Muscular pains occur with recurrent abdominal pains and a temporary edema. In the second week, inflammation of the muscle is the predominant symptom. Temperature may be 104° F. In several cases which we have observed, the symptoms are very suggestive of typhoid fever. A last period of encystment takes place with symptoms of marked cachexia, and edema of the face occurs. Delirium, somnolence, and lung affections take place. Either the patient dies at this time or the symptoms gradually subside. It is noteworthy that in all cases there is a marked eosinophilia. In a case observed (Gradwohl) eosinophilia was 90 per cent.

The disease occurs in nearly all countries of Europe and in America. There were widespread outbreaks in the old Kingdom of Prussia and some other parts of the German Empire. Here in the United States the incidence of this disease has been underestimated. Investigation during recent years has shown an increasing number of cases as determined by serial autopsies on cadavers as well as by clinical experiences.

"Regarding* meat inspection in this country, in reference to control of trichina infection in pork, microscopic inspection of pork for trichinae was discontinued in 1906. This inspection had been carried on in this country only on pork destined for the export trade and never done on any pork designed for domestic consumption. The inspection was inaugurated in 1891 because of certain restrictions placed on the importation of American pork by Germany and some other European countries. These restrictions had been largely removed by the year 1906 and this inspection was discontinued at that time. As a matter of fact, there never has been any microscopic inspection of pork intended for consumption within the United States. The Federal Meat Inspection Regulations provide for processing for the destruction of trichinae of products customarily eaten without cooking by the consumer. Such products include salami, mettwurst, Italian style hams, etc. This portion of the regulations therefore definitely provides protection against the acquisition of trichinosis from products of this sort which are produced in packing houses operating under these regulations. It may be said, however, that heretofore only about 70 per cent of the meat consumed in the United States has been packed under Federal inspection."

Riley and Scheifley examined the diaphragms of all cadavers in the dissecting room of the University of Minnesota and found evidences of trichinosis in 20 out of 117 cadavers. The most important means of communication from hog to man is through ingestion of pork products infested with this worm. Trichina infection in the hog depends almost entirely on the practice of feeding the swine garbage of raw or inadequately cooked pork scraps. Since human trichinosis is based almost entirely on feeding hogs raw garbage containing trichina-infested pork scraps, solution of the problem is clear. Garbage used in feeding hogs must be boiled for at least one-half hour, and all pork products must be properly processed before being offered for sale.

"Of their flesh shall ye not eat, and their carcass shall ye not touch; they are unclean to you."—Leviticus 11:8.

The earliest clue to the existence of trichinosis is the Mosaic injunction against eating pork.

Thorough cooking of pork constitutes the best method of prevention. Much would be gained in preventing the spread of this disease by an extension to small plants of the federal regulations regarding the preparation of sausages and other pork products which are ordinarily consumed without being cooked. It must be borne in mind that the usual process of pickling and smoking, even when long continued, does not destroy the Trichinellae contained in meat. Unless roasting is accompanied by thorough distribution of heat throughout the middle of the joint, the worm will not be killed. Naturally, all meat found infested on inspection should be condemned.

The autopsy findings in cases of trichinosis show definite cysts in the muscle. The muscles of the calves of the legs should be searched very carefully as well as other muscular parts where symptoms during life have suggested the presence of the cysts.

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*Personal communication. We are indebted for these precise facts to the courtesy of Dr. W. H. Wright, Chief, Division of Zoology, National Institutes of Health, Bethesda, Maryland.

It is necessary and advisable for the public not to purchase pork or pork products from any independent source unless the independent source has actual knowledge of freedom from infestation with this worm. A knowledge of this, of course, can be obtained only by a careful inspection of the slaughtered animals by an expert veterinarian.

Life Cycle.—The life cycle is given in Fig. 491. See page 1910.

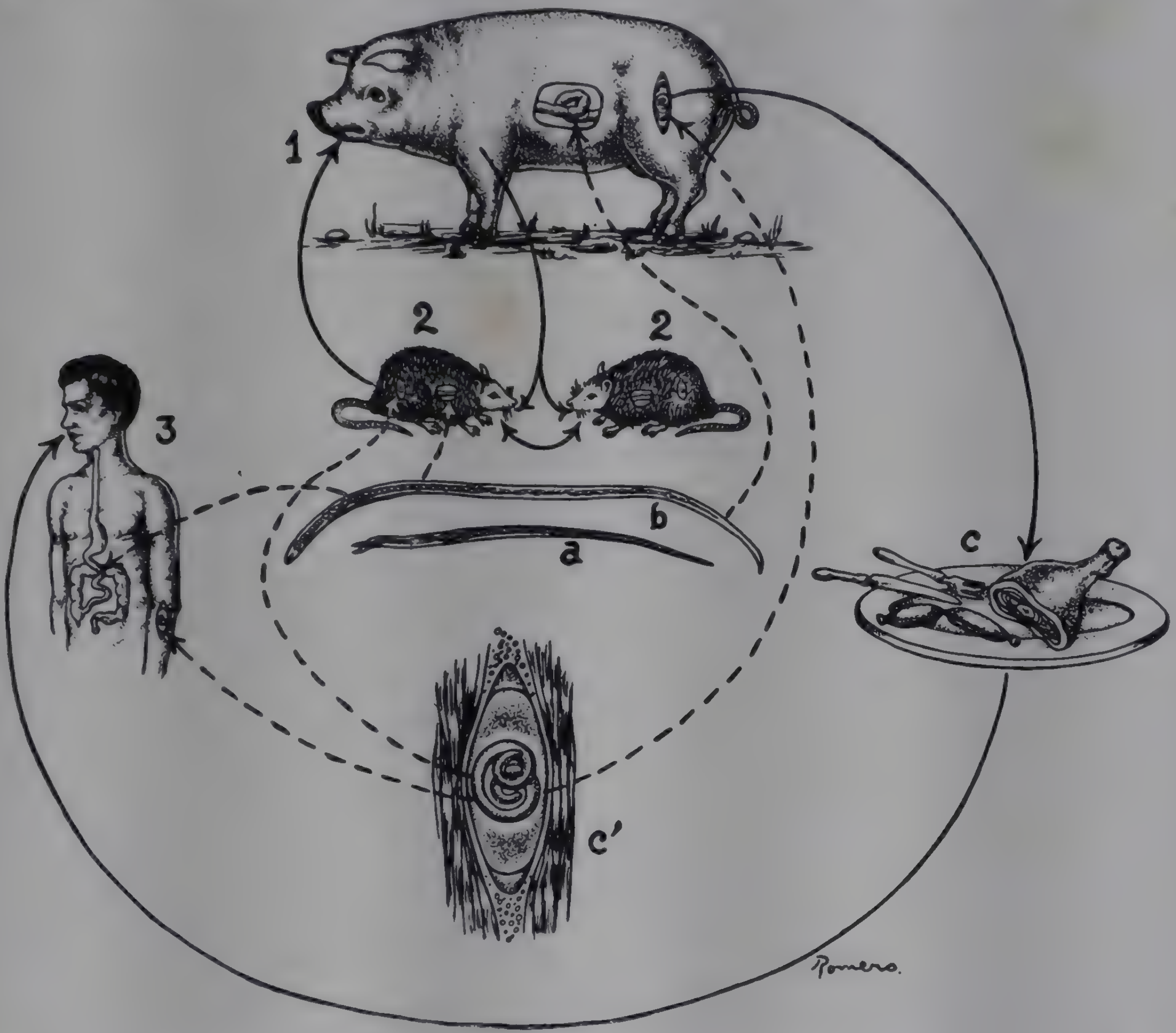

Fig. 491.—*Trichinella spiralis.* Life cycle. Numerous mammals may act both as definitive and intermediate hosts at the same time. The adult parasite, male (*a*) and female (*b*) live, normally, in the small intestine of the host; *1*, swine; *2*, *2*, rat; *3*, man; and others. After copulation, the female burrows in the intestinal mucosa, where it deposits the embryos which reach the circulatory system and migrate to the muscles where they encyst and develop into larvae (*c′*). Man (*3*) becomes infected when he ingests pork or its by-products (*c*), raw, poorly cooked, salted or smoked, contaminated with larval forms. Swine (*1*) usually become infected by ingestion of contaminated waste from other pigs, infected rats (*2*, *2*), or other contaminated animals. Rats become contaminated when they eat each other or when ingesting meat from another animal contaminated with the larval forms (*c′*). (Original drawing of Kourí and Basnuevo.)

Laboratory Diagnosis

Blood Picture.—The blood picture in the majority of cases shows definite leukocytosis, from 10,000 to 20,000. In some cases there is leukopenia. It is the presence of an eosinophilia that wakens the thought of trichinosis or some other worm disease. The eosinophilia may rise as high as 80 per cent; Gradwohl has seen a case with a percentage of 90.

Examination for Larvae.—Examination of the blood, spinal fluid, and feces for trichina larvae may be made. Positive findings are important although negative findings do not rule out trichinosis. Isolation of larvae is accomplished in only a small percentage of cases.

Biopsy is an inconvenient and painful procedure but not a dangerous one. By this is meant biopsy of muscle. Biopsies made before the twenty-first day after infection will be negative because the larvae will not have reached the muscles in sufficient concentration to be easily detected. Examinations are made by pressed preparations rather than by sections.

Intradermal and Precipitin Tests.—As well stated by Wright and Brady,[1] the development and refinement of these tests have provided additional valuable aids in the diagnosis of trichinosis. The antigen is prepared from powdered trichina larvae. As produced in the National Institutes of Health, the powder is extracted with neutral physiologic saline solution, the resulting material is sterilized fractionally by heat, and is dispensed in 2 c.c. hermetically sealed vials each containing 0.10 c.c., an amount more than sufficient for one intradermal test. Samples of this antigen exposed to sunlight and room temperature for a period of 6 months have shown no loss of potency when tested by the precipitin method, and presumably the antigen will retain its potency for even longer periods of time if stored in a refrigerator.

Tests with this antigen have indicated to date its species specificity in that it usually elicits positive reactions in patients infected with *Trichinella spiralis* but does not produce positive reactions in patients harboring such common intestinal parasites as *Ascaris lumbricoides, Necator americanus, Enterobius vermicularis,* and *Trichuris trichiura.* However, in using the antigen in suspected cases of trichinosis, it should be borne in mind that examinations made in the National Institutes of Health on approximately 4,900 diaphragms from unselected necropsies in various sections of the United States indicated that 16.4 per cent were positive for trichinae. If one person in six is infected with the parasite, it may be expected that some infected individuals will give a positive reaction to the intradermal test with trichina antigen even though their illness may not be related to clinical trichinosis. In fact, experimental evidence indicates the validity of this assumption. Furthermore, it should be borne in mind that tissue sensitivity to *T. spiralis* antigen may be present for years after a person has suffered from an attack of trichinosis and such persons may react positively to the intradermal test for relatively long periods of time. Under these circumstances, a positive reaction may mean that the patient is suffering from clinical trichinosis at the time of the test; that the patient has had in the past a trichina infection of a degree sufficient to produce persistent tissue sensitivity; or that the patient has at the time of the test a nonclinical trichina infection to which his present illness is unrelated etiologically. Considerable care should therefore be exercised in interpreting the results of the intradermal test. Due cognizance should be taken also of the fact that the test is usually not positive until after the second week of infection; if the test is applied earlier than this, the patient may fail to react. Positive reactions are usually of the immediate type and appear within 15 to 20 minutes after injection of the antigen. However, in exceptional cases, particularly in early stages of infection, there may occur a delayed type of reaction which does not reach its height before 24 hours.

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[1] Wright, W. H., and Brady, Frederick J., respectively Chief and Passed Assistant Surgeon, Division of Zoology, National Institutes of Health, United States Public Health Service. Monograph of August, 1940.

The precipitin test has the disadvantage of eliciting a positive response even later in the infection than does the intradermal test, since positive reactions are usually not obtained until after the third week of infection. However, circulating antibodies disappear before tissue sensitivity, and positive precipitin reactions are usually not obtained for longer than 1 to 2 years after infection. When possible, it is of advantage to make use of both tests in suspected cases, since every infected individual does not always react positively to each test. However, too much reliance should not be placed on these tests but rather the results should be given due consideration in connection with the clinical syndrome, the differential blood picture, the case history, and other factors. Occasionally individuals not infected with trichinae may react positively to these tests for reasons not apparent at the present time. It is possible that drugs and certain malignancies may influence the reaction.

In the past, many samples of serum submitted for this test have been unsuitable for use because they contained bacterial growth or hemolyzed blood, or were extremely chylous. If possible, the directions given below should be followed in obtaining and supplying samples of serum for this test.

1. Serum for the precipitin test should be secured either before, or 24 hours after, the application of the intradermal test.
2. The patient should be bled before breakfast in order to avoid chylous specimens and prevent deterioration of the sample en route.
3. All apparatus used for collecting specimens should be washed free of alkali and acid before sterilization.
4. Only sterile apparatus should be used.
5. The syringe used in withdrawing the blood should be rinsed with physiologic saline solution to prevent hemolysis.
6. The serum should be separated from the clot before hemolysis begins to take place, and rendered perfectly free from red cells and particles by centrifuging.
7. At least 2 c.c. of perfectly clear serum should be submitted; about 5 c.c. of blood will provide this amount of serum.
8. Do not heat or add preservative of any nature.

Complement Fixation Test.—The complement fixation test for trichinosis is considered quite reliable. The method of conducting the test for trichinosis is identical with that originated by Bozicevich for conducting a complement fixation test for amebiasis (see page 1831). The only difference is in the antigen. Wright[1] states that this complement fixation test for trichinosis is now used in his Department exclusively.

Preparation and Use of Trichina Antigen.—Bozicevich[2] has given full details for the preparation and use of an improved trichina antigen. This antigen is the same antigen recommended for diagnosis of trichinosis by precipitin and intradermal tests.

Obtain trichina larvae in the usual manner by infecting rats with trichinous meat and killing the rats 4 to 6 weeks after infection. Skin and eviscerate the rats, and grind the remainder of the carcasses in the meat grinder. The ground trichinous meat is then ready for digestion.

Hobmaier and Meyer[3] described a modification of the Baermann apparatus for the recovery of trichina larvae. For this purpose, the author prefers a funnel of 3-liter capacity, to the stem of which a centrifuge tube is attached by means of a short piece of rubber tubing. Place a pinchcock on the tubing to permit closing when the centrifuge tube containing the larvae is removed after digestion of the infested meat. Place a 6-inch perforated porcelain plate, such as is used in a desiccator, in the funnel, and over this plate lay 4 or 5 layers of cheesecloth having 40 to 44 mesh apertures to the inch.

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[1] Wright, W. H., Chief, Division of Tropical Diseases, National Institute of Health: Personal communication.

[2] Pub. Health Rep. 53: 2130, 1938.

[3] Science 86: 568, 1937.

Prepare the **digestive fluid** as follows:

Add 15 grams of pepsin to 3 liters of warm tap water. This amount is sufficient to digest approximately 70 grams of infested meat.

Stir until the pepsin dissolves.

Add 21 c.c. of hydrochloric acid, sp. gr. 1.10 to 1.19.

Place the digestive fluid in the modified Baermann apparatus (Fig. 492).

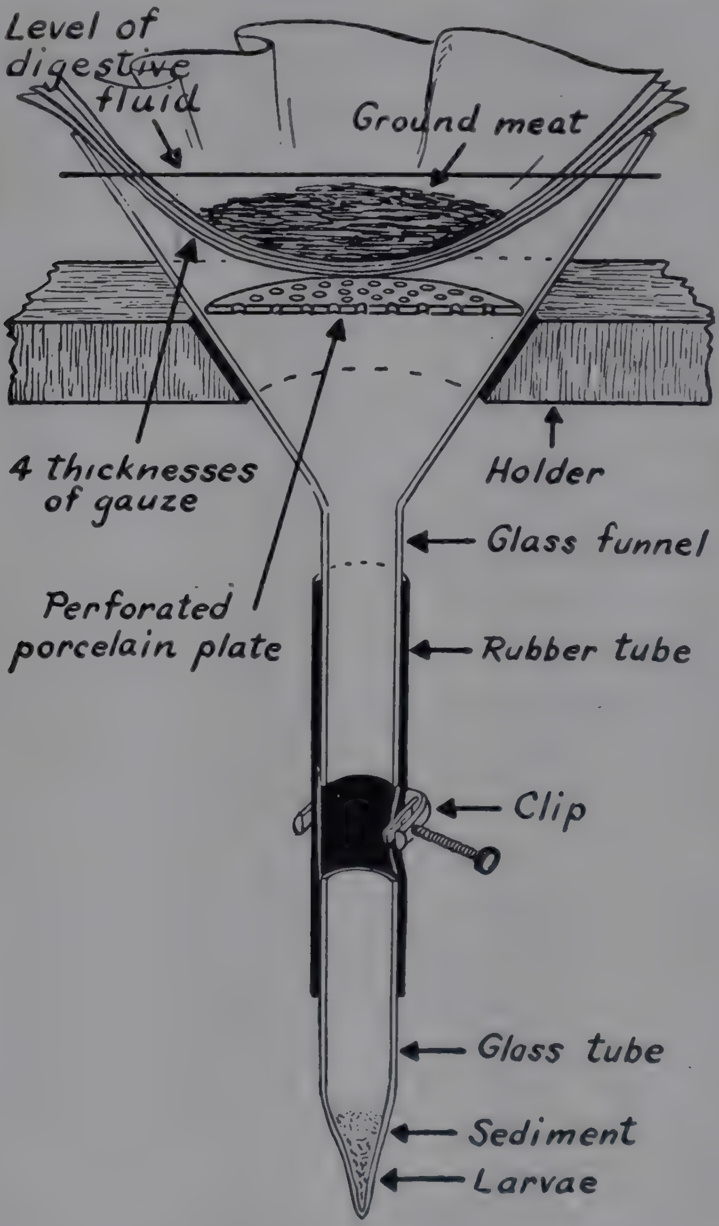


Fig. 492.—Modified Baermann apparatus.

Add the meat carefully, without agitation, on top of the cheesecloth.

Place the funnel containing these materials in an incubator at 37° C. for 15 to 18 hours. As the larvae are liberated from their cysts by the digestive fluid, they gravitate to the bottom of the centrifuge tube and, by means of their constant agitation, set in motion the fine sediment which settles over them when they become static. As a result, a clean, concentrated collection of larvae may be obtained, thus necessitating only a minimum amount of manipulation in removing rat protein.

At the end of the incubation period, close the pinchcock on the rubber tubing and remove the centrifuge tube.

Remove the supernatant fluid, along with the layer of sediment, by means of a pipette.

In order to neutralize the acid adsorbed from the digestive fluid, use a washing solution of pH 7.5. Wash the larvae three times with this washing solution over a 2-hour period. After each washing, allow the larvae to settle and draw off the supernatant fluid. After this, use neutral physiologic saline. At the end of this fourth washing, perform a biuret test on the supernatant fluid and, if the test is negative, the larvae are ready to be dried.

Place the larvae, with a minimum amount of fluid, in a sausage dialyzing skin 12 inches long and ½ inch in diameter. Place this in front of an electric fan. Evaporation is completed within 2 to 3 hours, depending upon the amount of fluid in the skin.

Then grind the larvae in an agate mortar. Since there is still considerable moisture remaining, place the mortar containing the material in a vacuum desiccator containing sulphuric acid or phosphorus pentoxide. After evacuation of the air, continue the drying overnight, and, when thoroughly dried, pulverize the mass and weigh it.

Calculate the extraction strength on the basis of the dried powder in a neutral 0.85 per cent solution of sodium chloride. A 1:20 dilution is usually the basic dilution used for precipitin work. After an extraction period of 3 to 4 hours at room temperature, adjust the reaction of the suspension to pH 7.0, if necessary, and continue the extraction in the refrigerator for 15 to 18 hours. Then centrifuge the suspension for one-half hour at the highest available speed. Discard the sediment.

Place the supernatant fluid in a water bath at 58° C. for 1 hour. Again centrifuge it and discard the sediment. Check and adjust the reaction, if necessary.

Set aside a portion of this fraction to be used in making **precipitin tests**, and dilute the remainder for **skin testing** in concentrations of 1:8,000 or 1:10,000, depending on the antigenic strength as determined by the precipitin titer given by a high titered antiserum. A high titered antiserum will give a good positive reaction with a 1:3,000 dilution of antigen. The solution used for skin testing is diluted to approximately three times the precipitin titer.

Distribute the antigen to be used for skin testing in 2 c.c. vials in quantities of 0.10 c.c., which is more than suffiicent for one intradermal test. Seal the vials hermetically in an oxygen flame. When they have cooled to room temperature, place them, sealed end down, in a beaker containing water stained with eosin or any suitable dye. Place the beaker containing the vials in a vacuum desiccator and apply house vacuum of 500 to 600 mm. In this manner, any defective seal will be detected readily when air is readmitted to the desiccator, since the stained water will enter any inadequately sealed tubes; such tubes should be discarded.

Check the seals, place the tubes in a water bath at 58° C. for one hour. Allow to stand at room temperature for 12 to 15 hours and place again in a water bath at 58° C. for one hour. Continue this fractional sterilization until sample tubes show no aerobic growth. Label and store the sterile tubes in a refrigerator.

Order **ASCARIDATA** Railliet and Henry 1915

Family **ASCARIDAE** Baird 1853

ASCARIASIS

Ascaris Lumbricoides Linnaeus 1758

Ascariasis is parasitism produced by *Ascaris lumbricoides*. It is found in every part of the world, but is more abundant in tropical countries because the high temperatures aid in the development of the parasite, and poor hygienic conditions of certain regions promote infestation.

Ascariasis is also known as ascaridiasis or roundworm infestation.

Morphologic Characteristics.—*Ascaris lumbricoides* is a large cylindrical worm tapering to pointed ends, the anterior end being thinner than the posterior. Pink while alive, it turns whitish when fixed with Formalin and yellowish if fixed with alcohol.

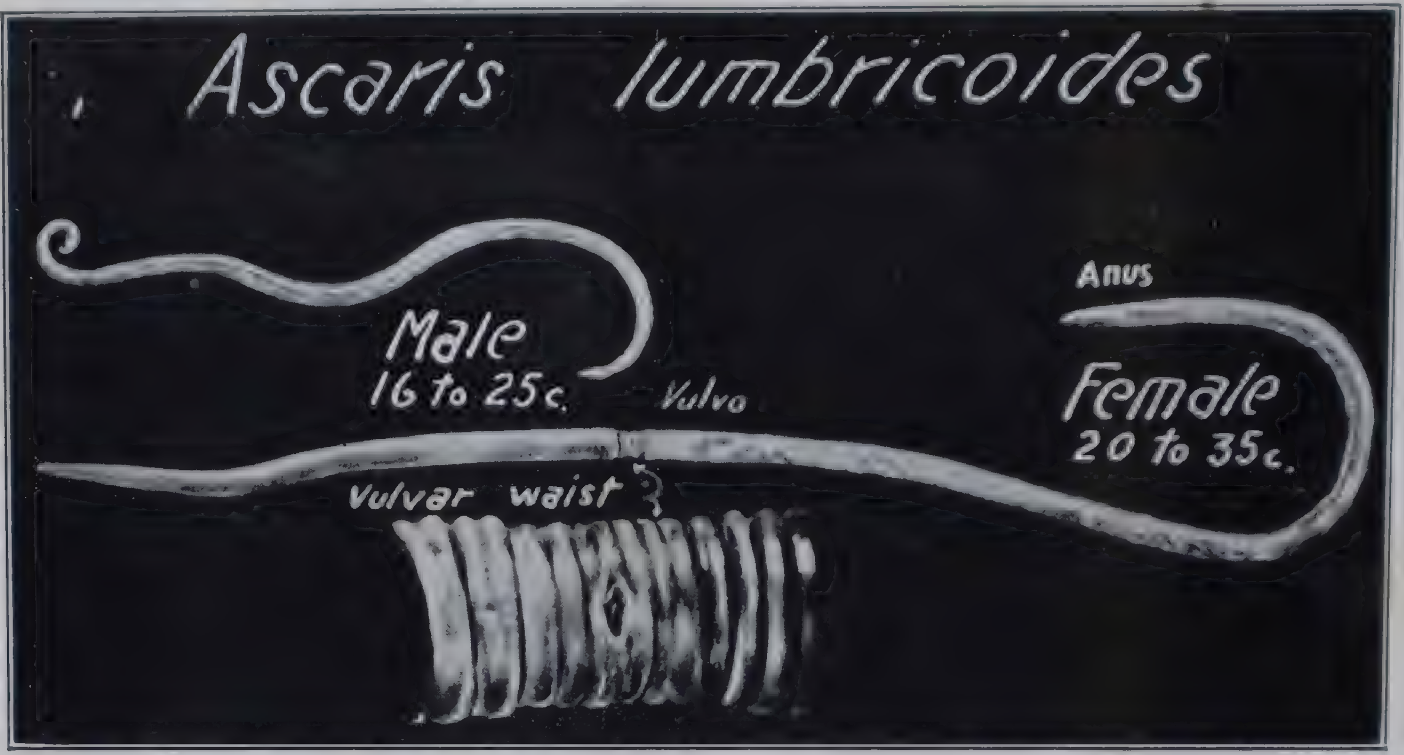

Ascaris lumbricoides
Anus
Male
16 to 25c.
Vulva
Female
20 to 35c.
Vulvar waist

Fig. 493.—Male and female *Ascaris lumbricoides,* natural size. Vulvar waist magnified. (Original illustration of Kourí.)

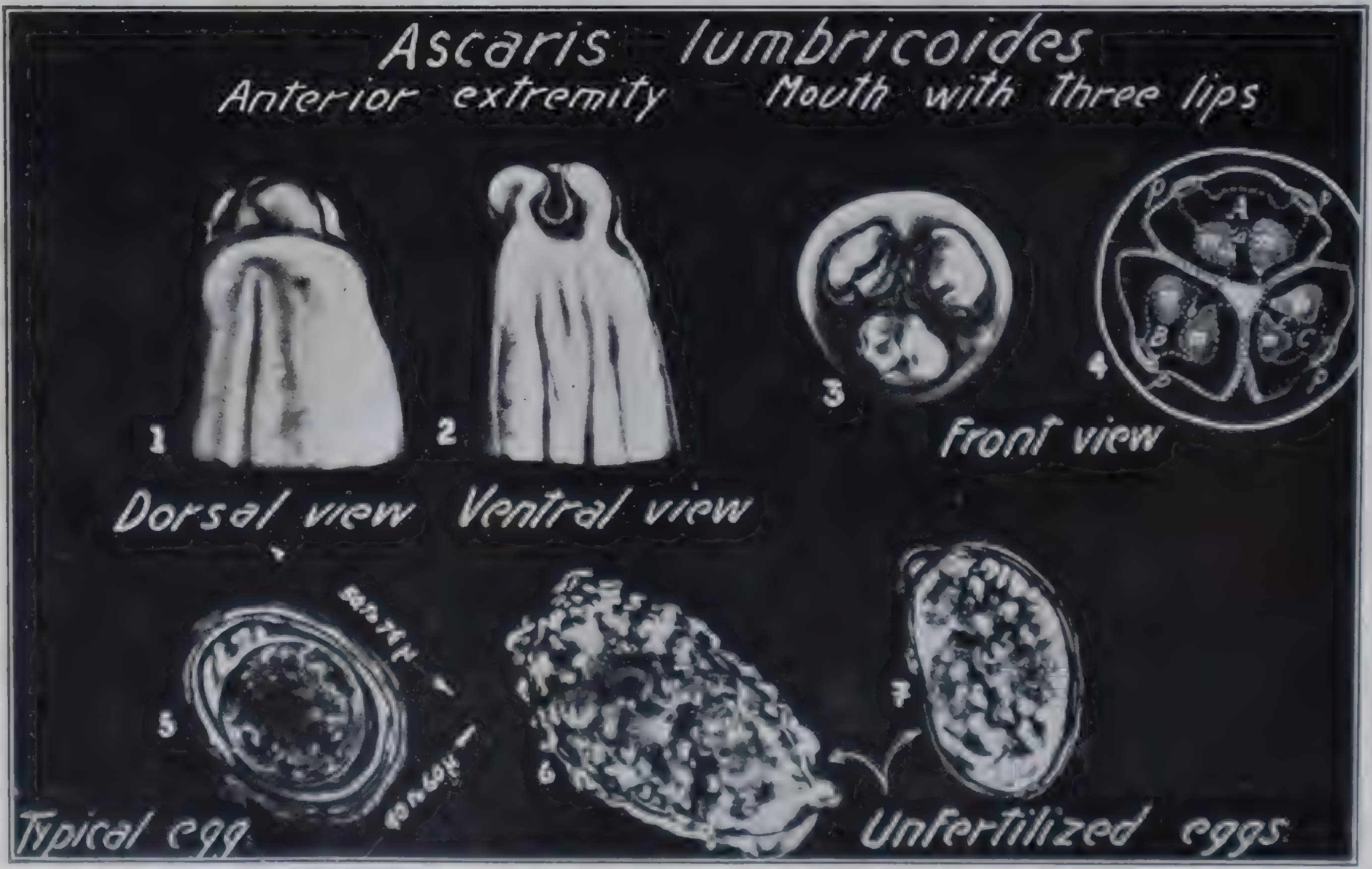
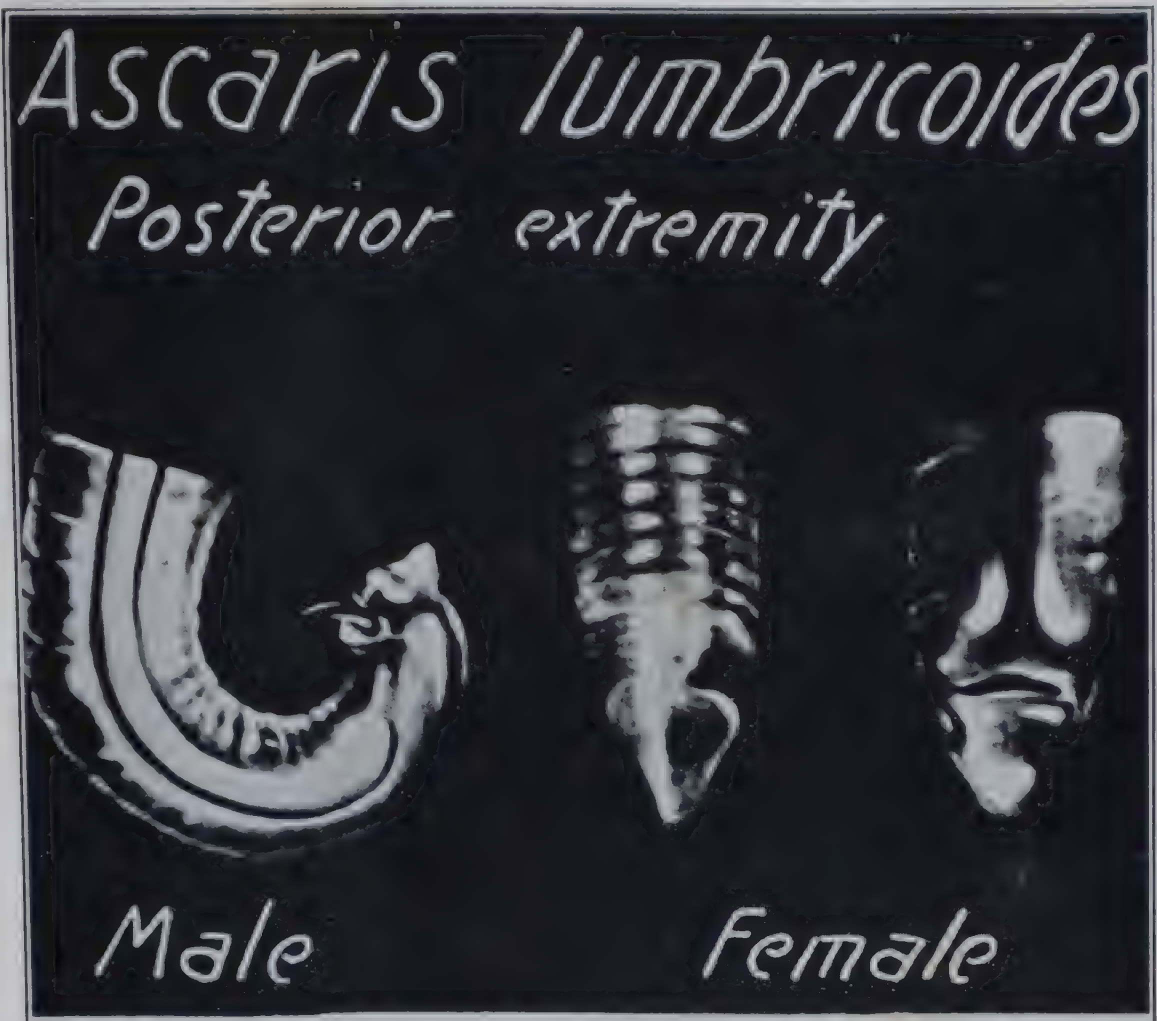

Ascaris lumbricoides
Anterior extremity
Mouth with three lips
1
2
3
4
A
B
C
P
Dorsal view
Ventral view
Front view
5
6
7
Typical egg
Unfertilized eggs

Fig 494.—*Ascaris lumbricoides.* *1* to *4,* Anterior extremity showing the mouth with three lips; *5,* fertilized (typical) egg; **6** and **7,** unfertilized (atypical) eggs. (No 4 is Guiart's sketch. The other drawings are after Kourí.)

Females are usually 20 to 35 cm. long, sometimes 40 cm., by about 5 mm. in breadth. Males are smaller, from 15 to 30 cm. in length and 2 to 4 mm. in the widest portion.

Fig. 495.


Ascaris lumbricoides
Posterior extremity
Male
Female

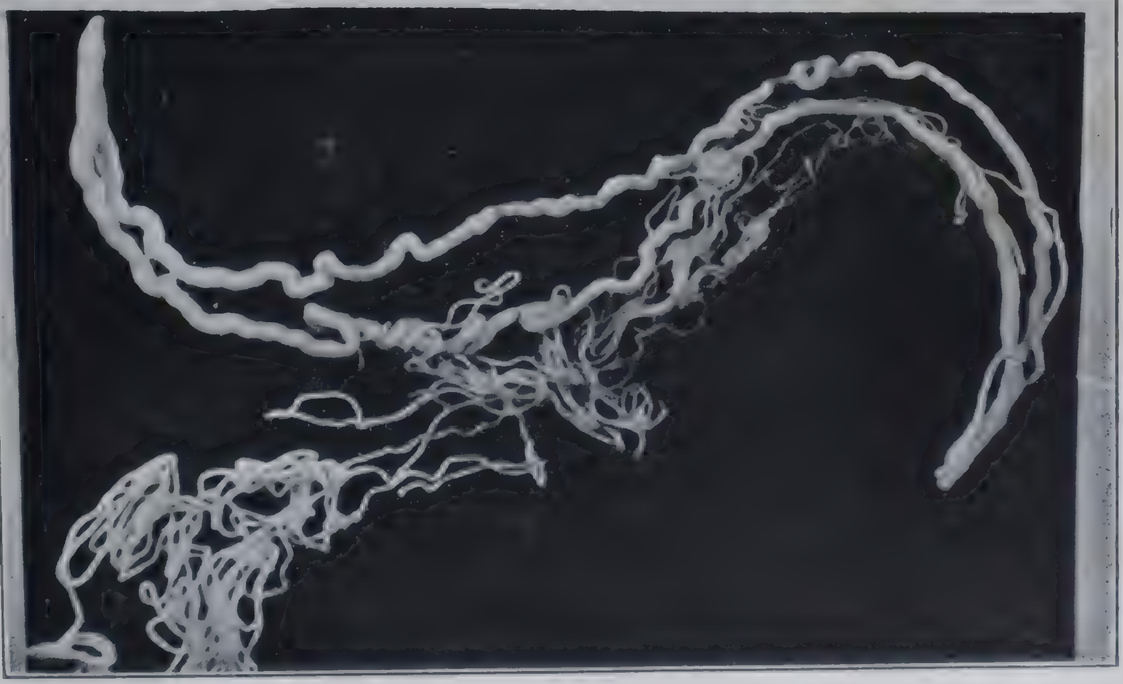


Fig. 496.

Fig. 495.—Posterior extremities of male and female *Ascaris lumbricoides*, drawn from life. (Original work of Prof. Kourí.)

Fig. 496.—Photograph of female genitalia. (Original work of Prof. Kourí.)

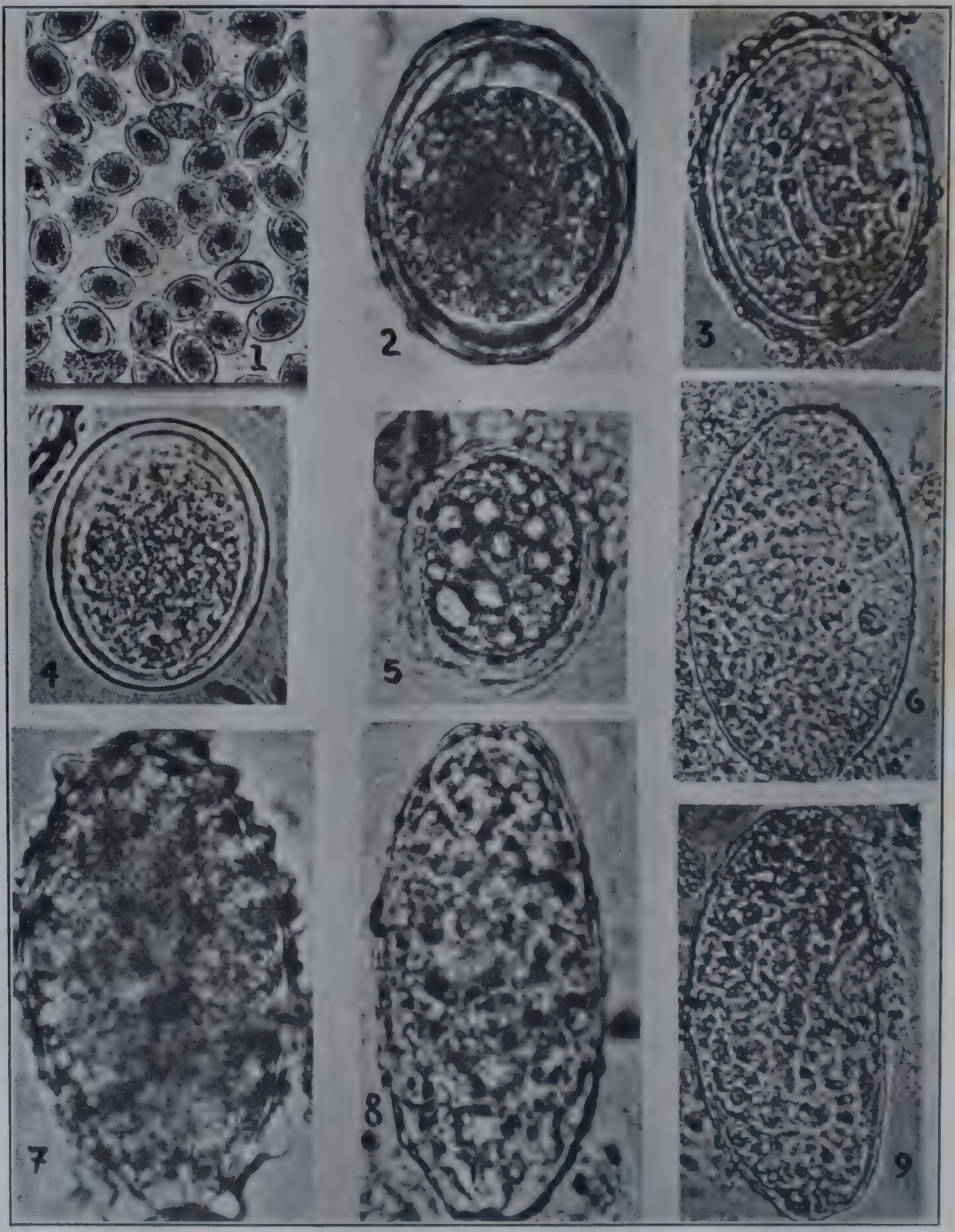


Fig. 497.—Photomicrograph of eggs of *Ascaris lumbricoides: 1*, Group of eggs removed from uterus; *2*, typical, or fertilized egg; *3* and *4*, eggs which have lost their external layer, *3* in part, and *4*, entirely; *5* to *9*, various unfertilized (atypical) eggs. (After Kourí.)

They are provided with a chitinous and resistant outer layer, transversely striated, and with two lateral narrow streaks running along the entire body length; these longitudinal lines correspond to the lateral fields. Female reproductive organs are often observed through the transparent parasite when viewed before a light.

A distinguishing characteristic of the male is the appearance of the posterior end, which curves ventrically.

Fertilized Eggs.—Eggs are ovoid with the longitudinal diameter longer than the transverse. They are 50 to 75 microns by 40 to 60 microns. Two membranes form the shell, the outer, festooned or mammillated, albuminous, stained more or less dark yellow with bile; and an inner smooth, stout, colorless, and refractive membrane. The two membranes are more in evidence when the egg is pressed between slide and cover glass, when it loses the mammillated outer covering.

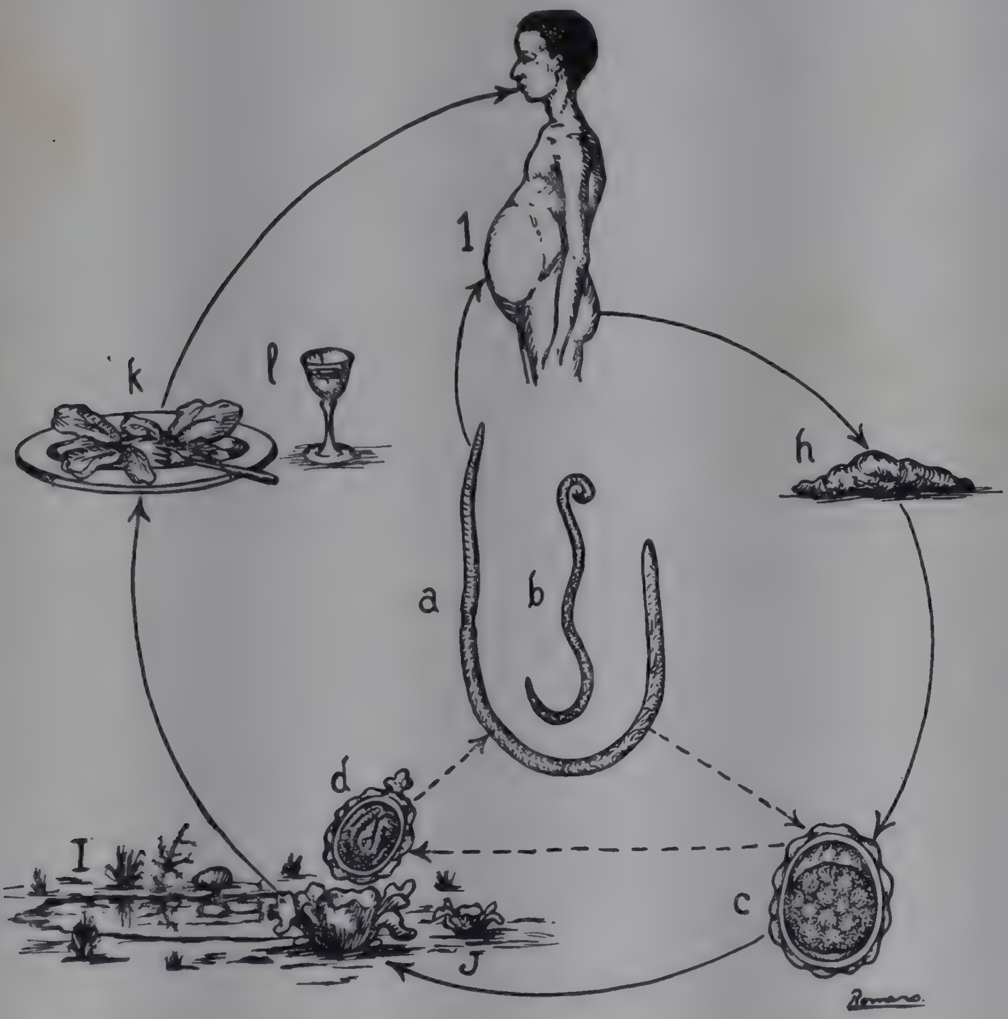


Fig. 498.—*Ascaris lumbricoides.* Life cycle. The fertilized eggs (*c*) are passed in the feces (*h*) of individuals (*1*) harboring female (*a*) and male (*b*) parasites; in two to three weeks the eggs embryonate (*d*); contaminate food (*i,j,k,l*) are ingested (*d*), develop into female (*a*) or male (*b*) adults in the intestines of their host (*1*), after migrating through the circulatory system; larval stages in the liver, the heart, and the lungs of their host (*1*). (Original drawing of Drs. Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

There is a spherical, sharply defined, granular protoplasm within the egg, but not occupying the entire egg cavity. It has a small, clear, rounded zone corresponding to the nucleus.

Unfertilized Eggs.—These are generally longer than the fertilized eggs although some of them are small. The shell is much thinner. The mammillated shell disappears with slight pressure. The contents consist of large refractile granules resembling fat droplets.

Life Cycle.—The life cycle is seen in Fig. 498.

Habitat, Number, and Life Span.—The usual habitat of this parasite is the small intestine of man. In erratic parasitism it may be found in any other part of the digestive tract. The number of parasites ranges from one to two hundred; in exceptional cases there may be more than one thousand; usually there are only eight or ten.

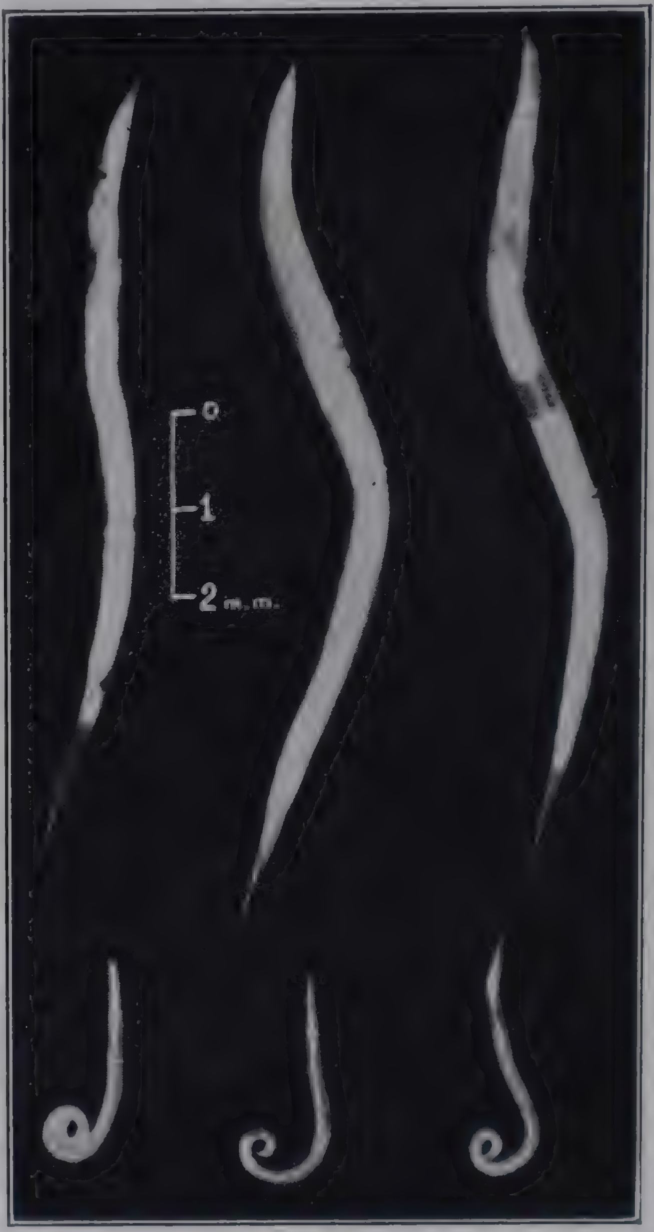


Fig. 499.—*Enterobius vermicularis.* Above, three females, showing the characteristic fine, long tail; below, three males, much smaller, with the posterior extremity coiled upward. (Original figure of Kourí and Basnuevo.)

The life span of this parasite is unknown.

Ascaris lumbricoides which infests man is morphologically identical to *Ascaris suum* of swine; biologically, however, they are different species. Koino, in 1922, and other more recent investigators have demonstrated that the Ascaris which parasitizes man cannot be transmitted to swine, and vice versa.

Diagnosis.—The only definitive diagnosis consists in finding the parasite or its eggs. Direct microscopic examination of feces and the use of concentration methods constitute the usual procedure in the diagnosis of this parasitism. The characteristics of unfertilized eggs are important in avoiding diagnostic errors. Consecutive examinations at several days' intervals should be practiced.

Parasitism by male Ascaris cannot be diagnosed by the microscopic examination of feces alone. Test treatments and occasionally x-ray examinations might prove diagnosis in cases of this type.

In gastroduodenal and biliary localization of the parasite, eggs are found in microscopic examination of fluids obtained through duodenal intubation.

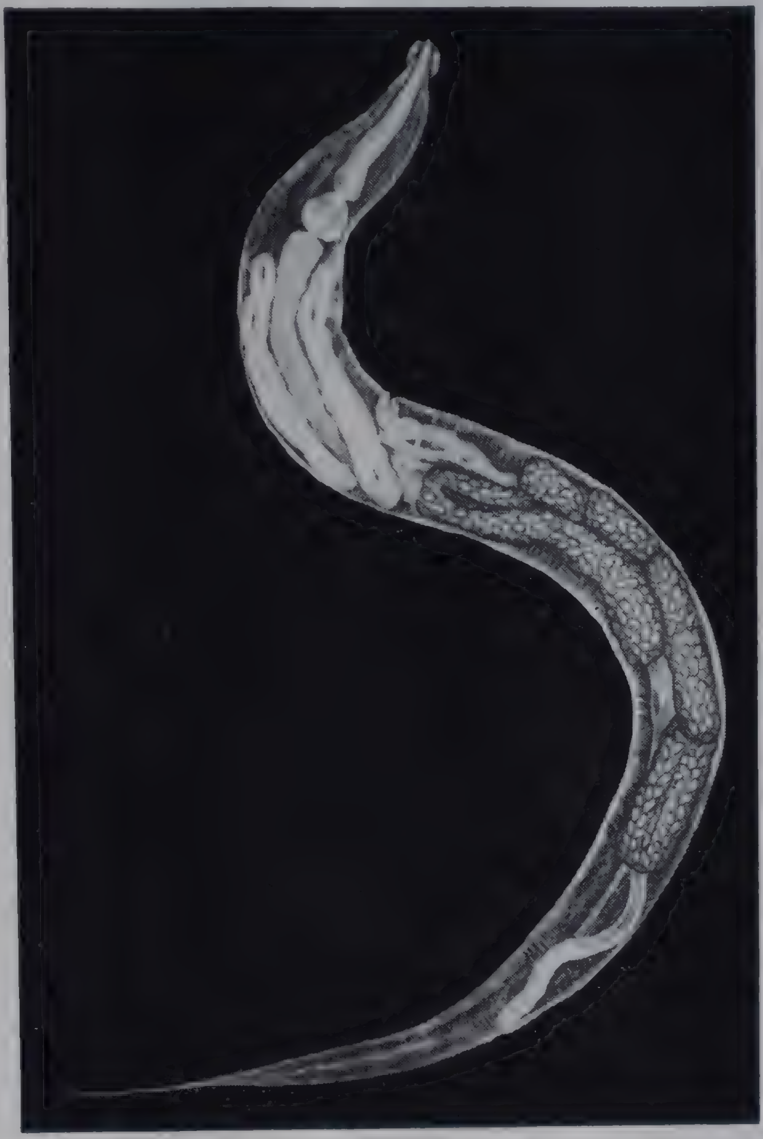


Fig. 500.—*Enterobius vermicularis.* Pregravid female parasite. Observe the cuticular cervical expansions, the muscular esophagus, with its two portions, the intestine, ovaries, uterus, vulva, and anus. (Original figure of Kourí and Basnuevo.)

Family **OXYURIDAE** Cobbold 1864

ENTEROBIASIS (OXYURIASIS)

Enterobius Vermicularis (Linnaeus 1785) Leach 1853

Enterobiasis, or pinworm infestation, is parasitism produced by *Enterobius vermicularis. E. vermicularis* is a very small worm, of a whitish color. The posterior extremity of the female is very long and sharp, that of the male conical and coiled. The male measures 2 to 5 mm. in length and the female 9 to 11 mm. The females characteristically have a fine, long, pointed tail.

The **eggs** are oblong, asymmetrical, flat on one side and convex on the other. They are about 60 microns long and approximately 30 microns wide. In addition to being asymmetrical, they are double-walled, colorless, and contain a tadpole-shaped or worm-shaped embryo. The embryo emerges from the cephalic pole or adherence zone of the egg, the convex end portion. The eggs are embryonated when laid. They often adhere to each other, forming a cluster.

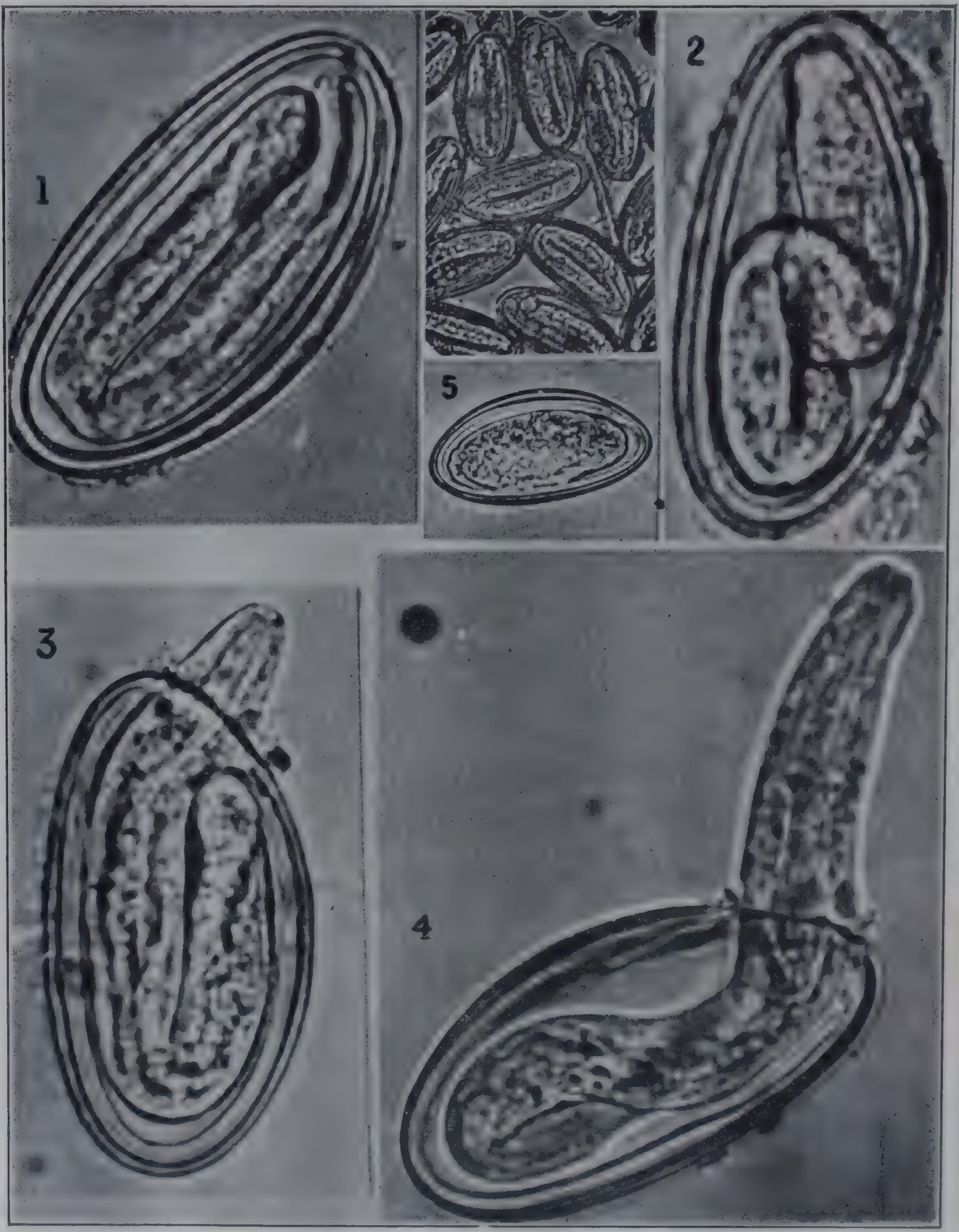


Fig. 501.—Eggs of *Enterobius vermicularis.* *1* and *2*, Egg with vermiform embryo. *3* and *4*, Two stages in the emergence of the vermiform embryo. *5*, Eggs with lower magnification. (Original photomicrographs of Kourí.)

Life Cycle.—The life cycle of this worm is set forth in Fig. 502.

Diagnosis.—When infestation with *E. vermicularis* is suspected, the best diagnostic procedure is to ask the patient if he has noticed any small whitish worms in the feces. If the answer is in the affirmative, direct him to bring a specimen in a small bottle containing water, alcohol, or 10 per cent Formalin. Other methods of diagnosis are parasitologic examination of the feces, microscopic examination of the scrapings of the marginal regions of the anus, examination of the cellophane covering of Hall's anal swab (see page 1813), and microscopic examination of material from the subungual groove.

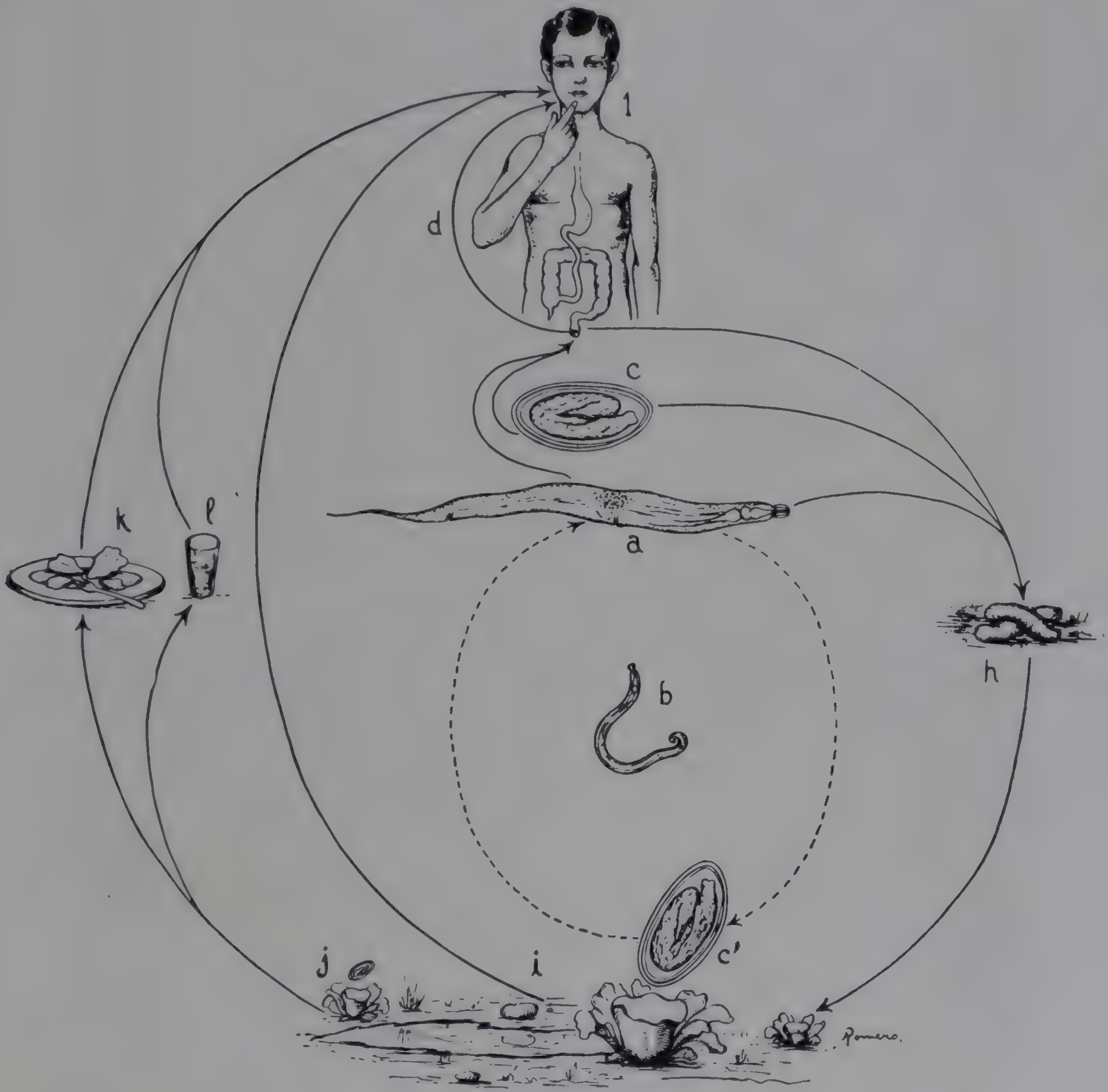


Fig. 502.—*Enterobius vermicularis.* Life cycle. Auto-infestation (*d*) occurs because the egg is embryonated and infestive when first laid in the anal margins or outside the body. *a*, Oviferous female in the anal margins, lays it eggs, *c*, which contaminate the fingers of the host, or, in the feces (*h*), drop on the soil, *c'*, and contaminate foodstuffs, *k,l*. When the eggs are ingested, the intraovular embryo is set free in the intestines, where the adult female *a*, or male, *b*, develops. After copulation the males, *b*, remain in the cecum and die. The oviferous females, *a*, descend to the anal margins of the hosts, *1*, to again begin the cycle. (Original figure of Kourí and Basnuevo.)

Finding the egg is essential for the diagnosis, but eggs are not found constantly in the feces of parasitized individuals, since the female parasite does not lay eggs in the intestine of the host, but migrates to the rectum and anus and lays the eggs there, or empties its uterus after leaving the body of the host. It is for this reason that the Hall anal swab procedure is so important in the diagnosis of this parasitism.

Family **RHABDITIDAE** Micoletzky 1922

STRONGYLOIDIASIS

Strongyloidiasis is parasitism produced by *Strongyloides stercoralis.*

Strongyloides Stercoralis (Bavay 1876) Stiles and Hassal 1902

S. stercoralis has two adult forms and a rather complex cycle of development. There are two types of larvae, one the rhabditiform, the other filariform. The first adult form is called "strongyloid" because of the shape of the esophagus which is cylindrical and long. The second form is called "rhab-

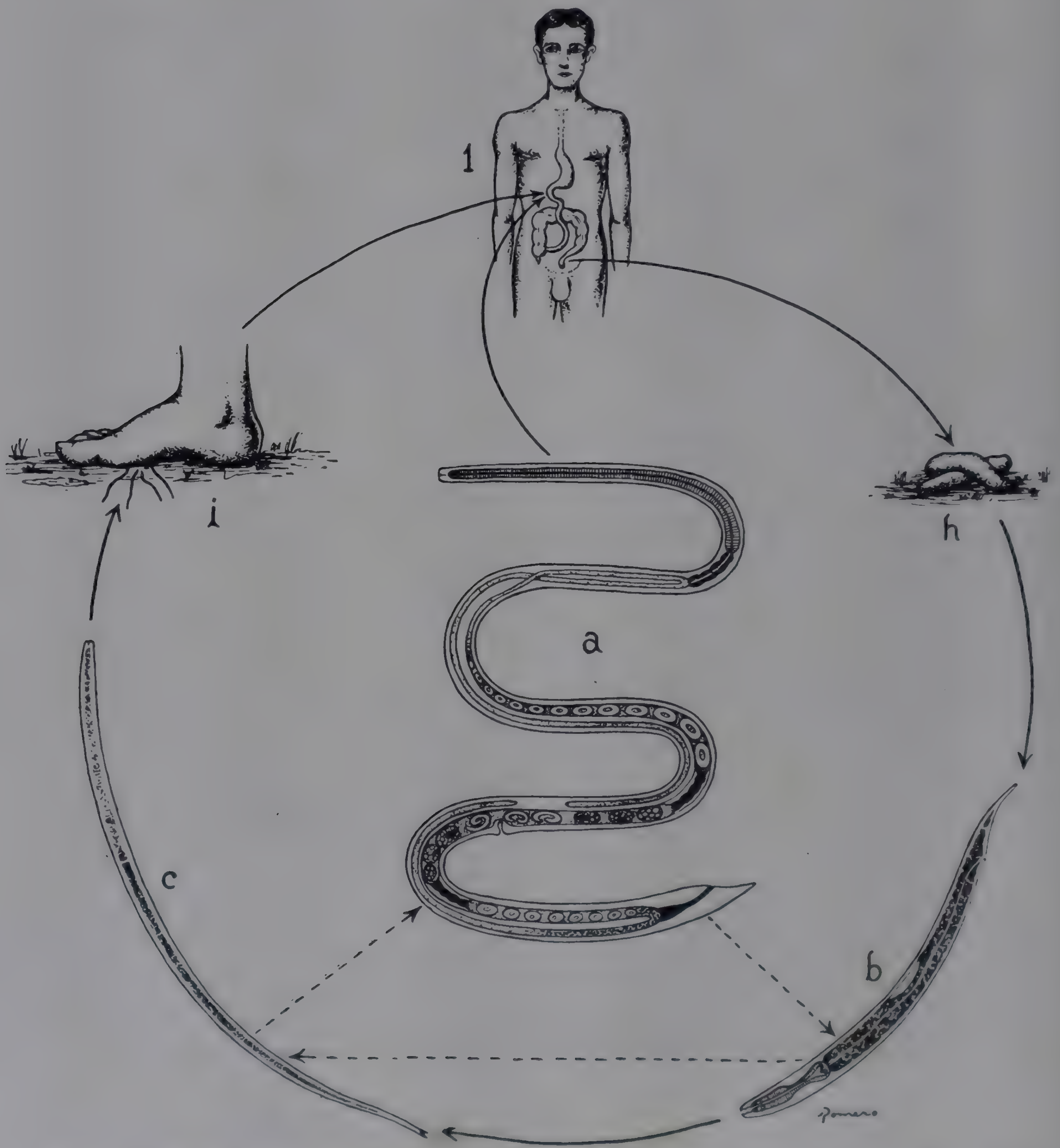


Fig. 503.—*Strongyloides stercoralis.* Life cycle. Direct or short cycle. The female parasite (*a*), in the intestinal mucosa of the host (*1*) lays eggs which burst and free, in the feces (*h*), a rhabditiform larva (*b*) which is transformed directly into a filariform larva (*c*) which bores through the skin (*i*), migrates to the intestines, and is transformed into the parasitic form (*a*), which adheres to the mucosa, lays eggs, and begins anew its cycle with freeing of the rhabditiform larvae (*b*) in the feces (*h*). (Original figure of Kourí and Basnuevo.)

ditiform,'' because of the bulbous shape of the esophagus. At the time of expulsion, the rhabditiform larva is 200 to 300 microns long, and rapidly increases in size as it develops into the filariform larva.

The **rhabditoid female,** fecal or free type, is about 1 mm. long and 50 microns wide, and can be seen with the naked eye. If a drop of coproculture is placed on a slide, it can be seen but is difficult to detect because of its small size.

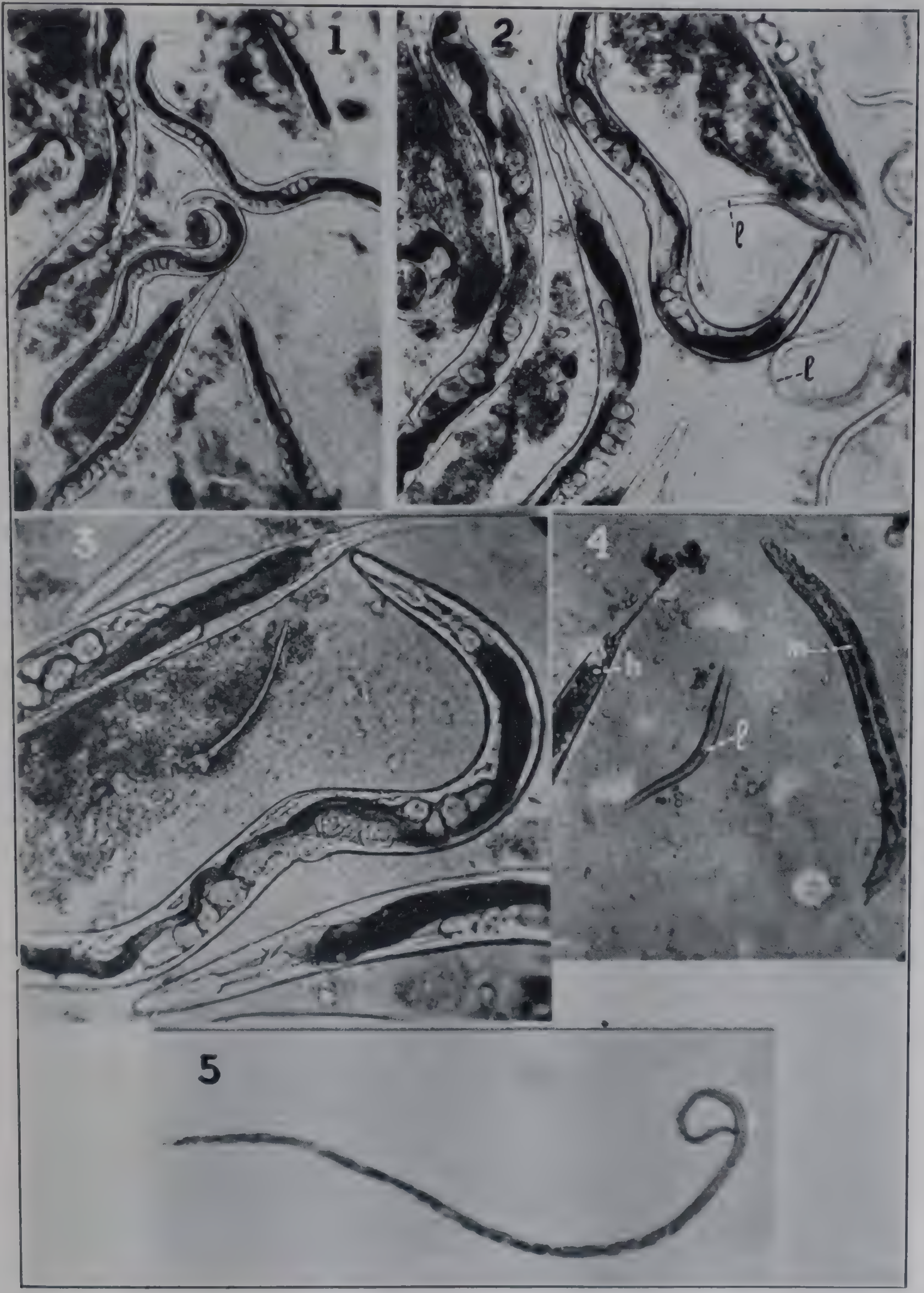


Fig. 504.—*Strongyloides stercoralis.* *1,* Gravid rhabditoid females in fecal cultures *2,* Free-living females and strongyloid larvae in fecal cultures. *3,* Rhabditoid free-living females, higher magnification. *4,* Posterior extremity of female, *h,* rhabditiform larva, *l,* and free-living male, *m.* *5,* Female parasite, Strongyloides, from a rat. (Kourí and collaborators.)

The **eggs** are ellipsoid and measure approximately 50 to 60 microns in length by 30 to 40 microns in width. They have a very thin single, colorless, refractile wall. In fecal cultures, some of these eggs burst, giving rise to rhabditiform larvae within the uterus of the rhabditoid female.

The **life cycle** is shown in Fig. 503.

The **rhabditoid male** is seen in Fig. 504.

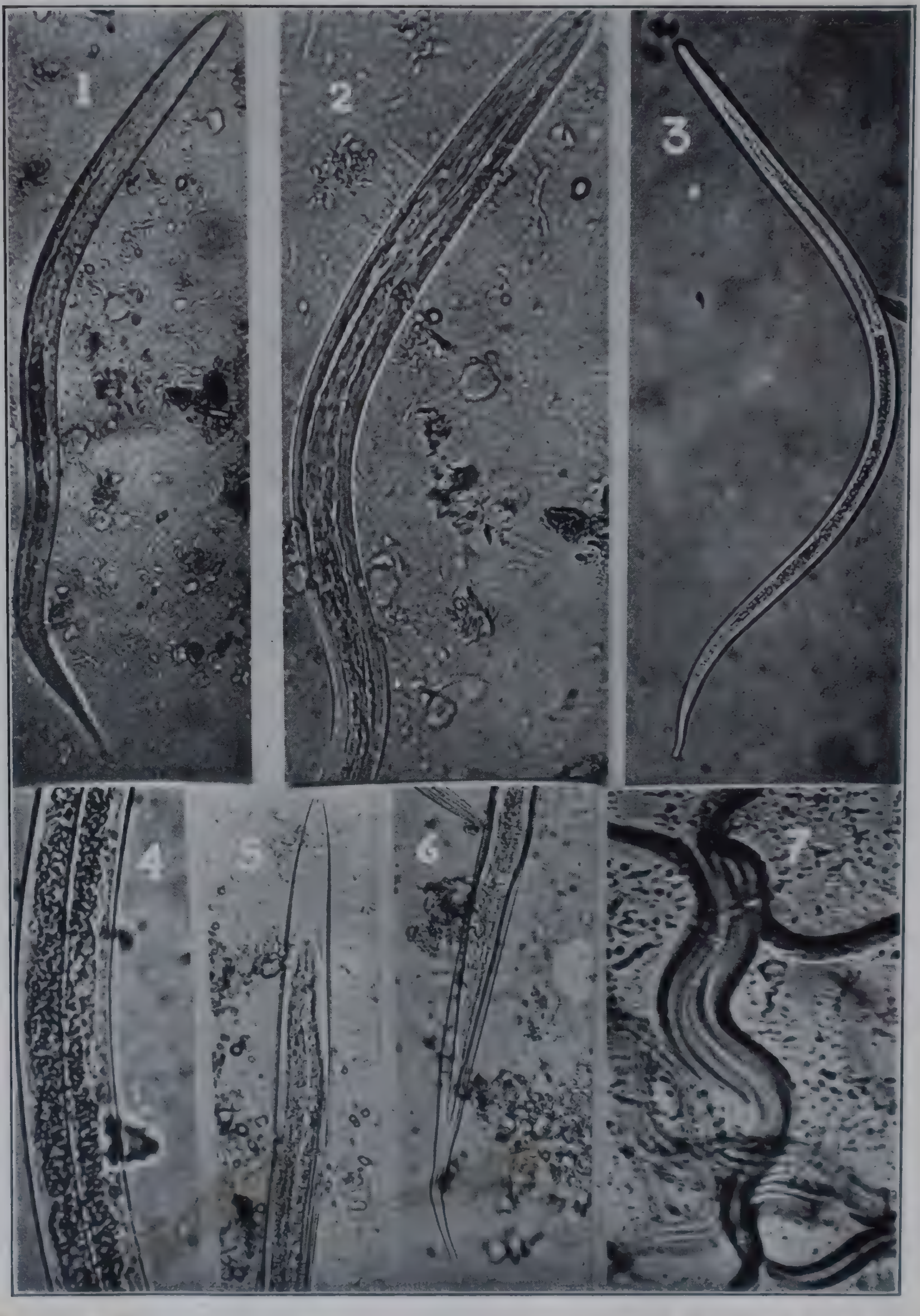


Fig. 505.—*Strongyloides stercoralis*. Developing larva and filariform larva in coprocultures. *1*, Larva developing into filariform larva. The esophagus has become longer and is almost cylindrical; the intestine has become shorter; and the outline of the genital organs has narrowed. *2*, Anterior half of the same larva under high magnification. *3*, Typical filariform larva. *4*, Central portion of body of larva developing into filariform. *5* and *6*, Anterior and posterior portions of larva during molting stage. *7*, Group of filariform larvae (arranged like locks of hair) in the coprocultures. (After Kourí and collaborators.)

Diagnosis.—Diagnosis is established by microscopic examination of the feces, where the rhabditiform larvae are found. If the feces are not examined for some time after they have been passed, the filariform larvae, and even sexual forms, might be seen.

Stools are examined unstained, stained with Lugol solution, concentrated, or by screening. Coproculture is important when parasites are scarce. Examination of bile obtained by duodenal intubation may show rhabditiform larvae or embryonated eggs surrounded by mucus.

Order **STRONGYLATA** Railliet and Henry 1913

Family **ANCYLOSTOMIDAE** Lane 1917

HOOKWORM DISEASE

Hookworm disease is parasitism produced by *Necator americanus* or *Ancylostoma duodenale.*

Necator Americanus (Stiles 1902) Stiles 1903, and **Ancylostoma Duodenale** (Dubini 1843) Creplin 1845

The species *Necator americanus* predominates in the Western Hemisphere, Central South Africa, Southern Asia, East India, Polynesia, Micronesia, and Australia. *Ancylostoma duodenale* predominates along the coastal regions of Northern Africa, the Mediterranean, Southern Europe, Northern India, and China and Japan.

N. americanus is a pinkish-white round worm, frequently stained dark by digested blood. The female is 9 to 11 mm. long and 0.4 mm. wide, the male 7 to 9 mm. long by 0.3 mm. wide. The body of the female describes an arc, the ventral surface on the inner side. The anterior surface curves back over the body with the buccal capsule facing the convex dorsal surface.

TABLE 184

ANCYLOSTOMA DUODENALE	NECATOR AMERICANUS
Buccal Capsule	
Note teeth. Two on each side.	Note absence of teeth. Instead of this, there are liplike pouch-formed plates.
Distal End of Female	
A very fine sharp point or tail.	No tail or point.
Distal End of Male	
Note spicules which come off from the bursa copulatrix diverging from each other and ending in a point. The bursa is spread out in umbrella shape.	The spicules which come from the bursa copulatrix lie near the end and close together and carry a hook. The bursa appears as if it were made of two lobes.
Sexual Opening of the Female	
Sexual opening lies behind the middle of the body in the convexity.	Sexual opening lies in front of the middle of the body in concavity.
Position of the Body in Rigor Mortis	
The head, and in the male the distal end, are bowed in from the ventral side of the body. ("C" position.)	The head, and in the male the distal end, are bent from the dorsal side almost hook shape.
Size	
Somewhat larger and thicker than the Necator.	Somewhat shorter and more slender than the Ancylostoma.

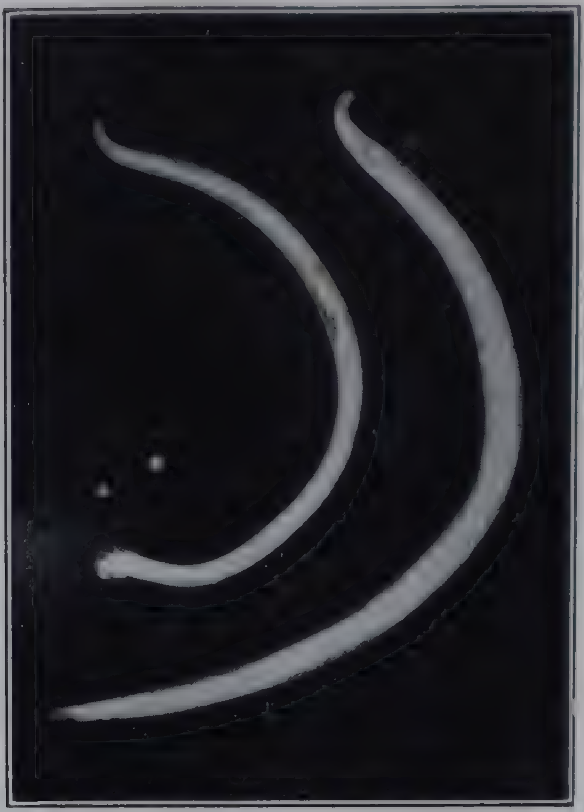


Fig. 506.—*Necator americanus. Left,* male. *Right,* female. (Original figure of Drs. Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

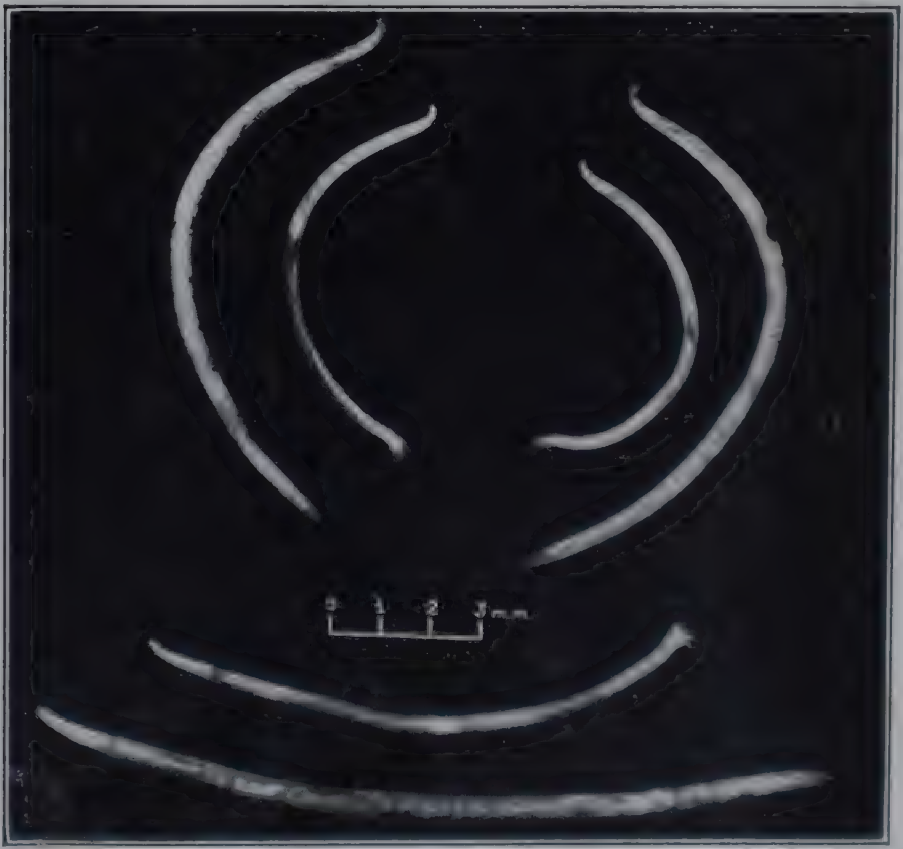


Fig. 507.—Necator and Ancylostoma. *Above, Necator americanus,* male and female (two pairs); *below, Ancylostoma duodenale,* male and female (one pair). Millimeter scale. (Original figure of Kourí.)

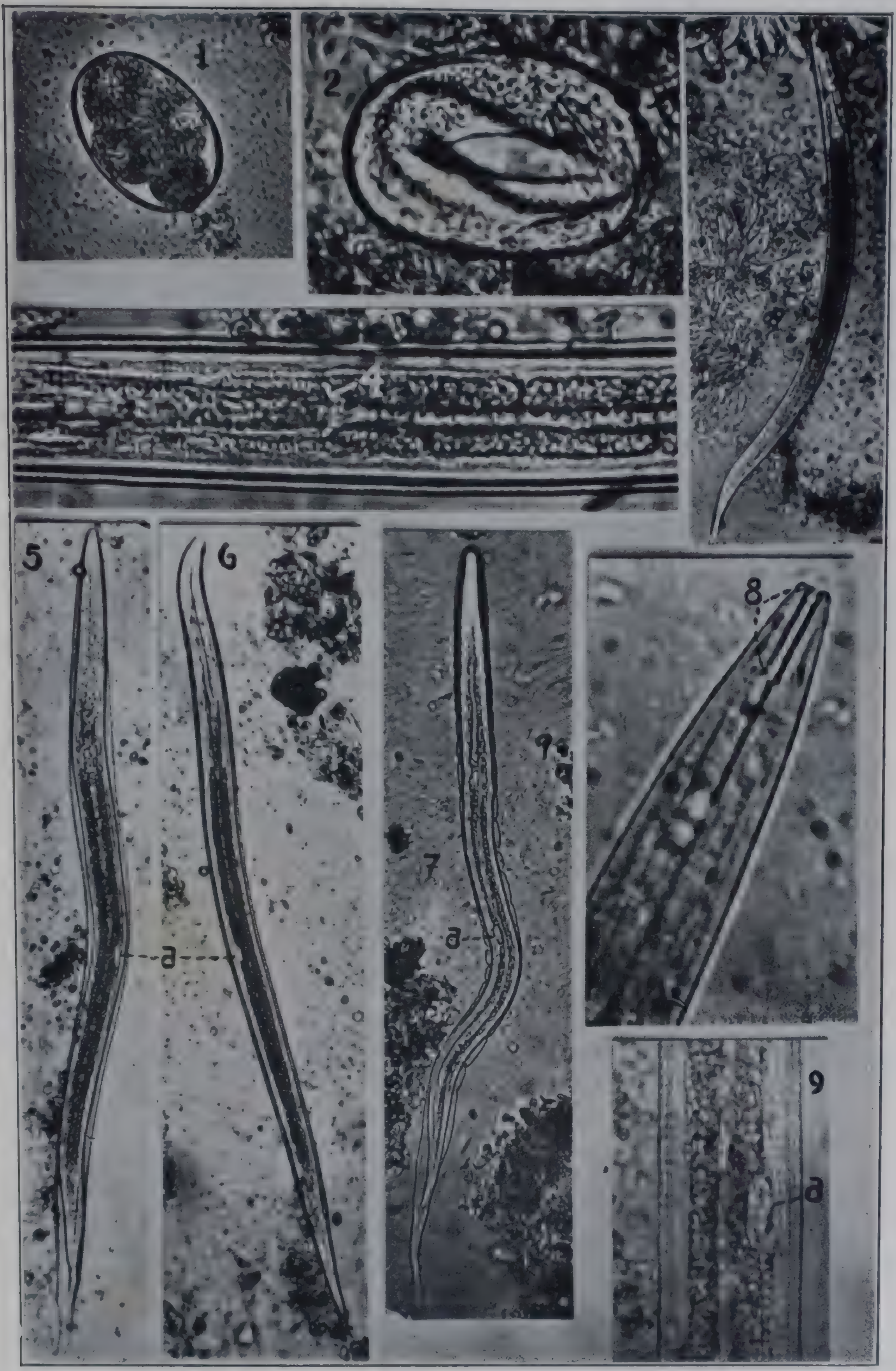


Fig. 508.—*Necator americanus,* eggs and larvae. *1,* Eggs with 4 segments or cells; *2,* embryonated egg with long mouth; *3,* rhabditiform larva, esophagus with 2 dilated portions, rudiments of genital organ not visible; *4,* point of junction of esophagus, left, and intestine, right, of filariform larva; *5, 6,* and *7,* various developmental stages of filariform larva; *7,* sheathed filariform larva, infesting form with (*a*) rudiment of small genital organ; *8,* long mouth of rhabditiform larvae; *9a,* small rudiment of genital organ in rhabditiform larva. In 5 and 7 the sheath is clearly outlined. (Original photomicrographs of Kourí.)

Female *A. duodenale* species are 10 to 18 mm. long by 0.6 mm. wide; males are 8 to 11 mm. long and 0.4 mm. wide. Ancylostoma shows an irregular curvature.

The females of the two worms differ in size and shape, and the buccal capsules. See Table 184. In the *N. americanus,* the vulva is in the median third of the body; in the *A. duodenale,* it is in the posterior third.

Eggs.—Eggs of *N. americanus* are ellipsoidal, and are longer than those of *A. duodenale,* which measure 60 microns by 40 microns. Eggs of *N. americanus* are 70 by 40 microns, the wall is very thin, colorless, refractile. The eggs are composed of a granular grayish substance divided into 4 to 8 cells or metameres (blastomeres), each containing a clear, rounded zone which represents the nucleus.

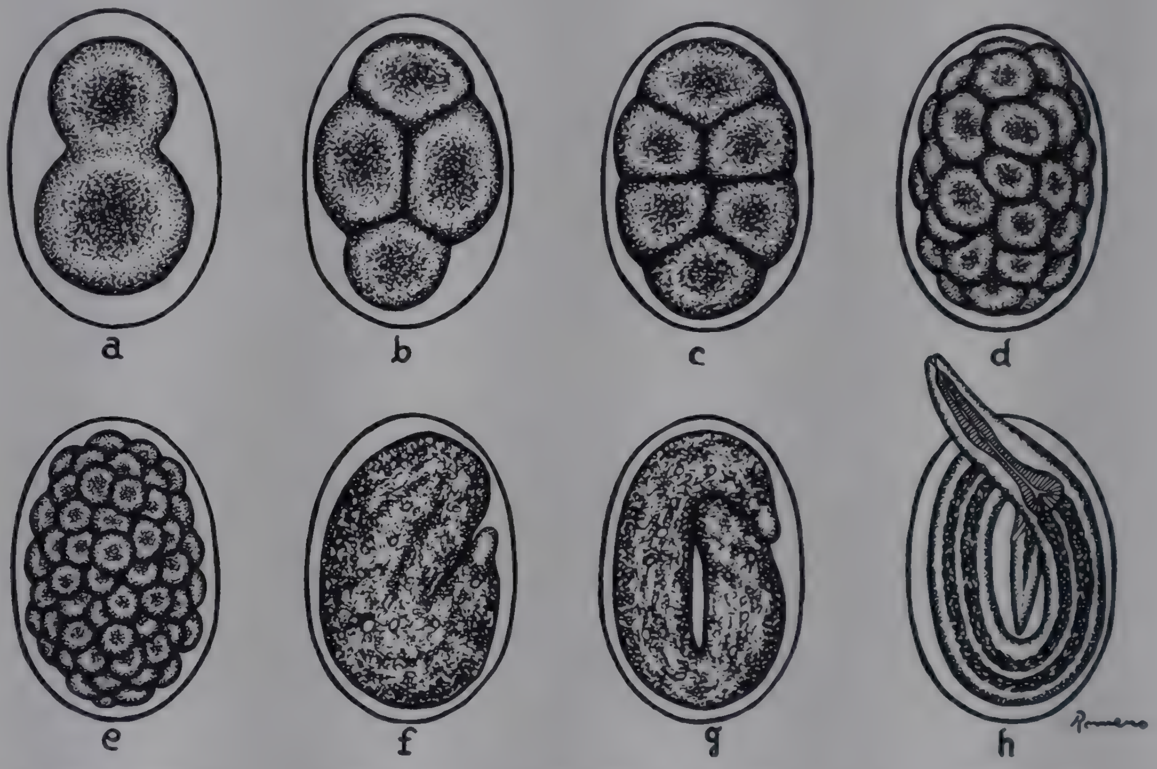


Fig. 509.—*Necator americanus.* Development of the egg. *a, b, c,* Eggs with two, four, and six metameres; *d,* beginning of the morula stage; *e,* morula in a more advanced stage of development; *f,* egg with tadpole-like embryo; *g,* egg with vermiform embryo; *h,* egg with rhabditiform larva. (Original drawing of Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

Life Cycle.—The life cycle is given in Fig. 510.

N. americanus and *A. duodenale* live in the small intestine of man, especially in the upper portion. The number of worms harbored by some patients may be in the thousands in cases with severe anemia. The life span of the parasite is probably from 1 to 5 years.

Hookworm disease shows various symptoms: cutaneous, intestinal, and general. For a description of hookworm anemia, see page 750.

Diagnosis.—Diagnosis is often confused with malarial cachexia or beriberi. Confirmatory diagnosis should be made by examination of the feces, where the eggs are numerous in cases of massive parasitism. Concentration methods are necessary in cases of mild infestation. Study of the duodenal contents obtained by intubation with Lyon's catheter may reveal both eggs and adult forms of the parasite.

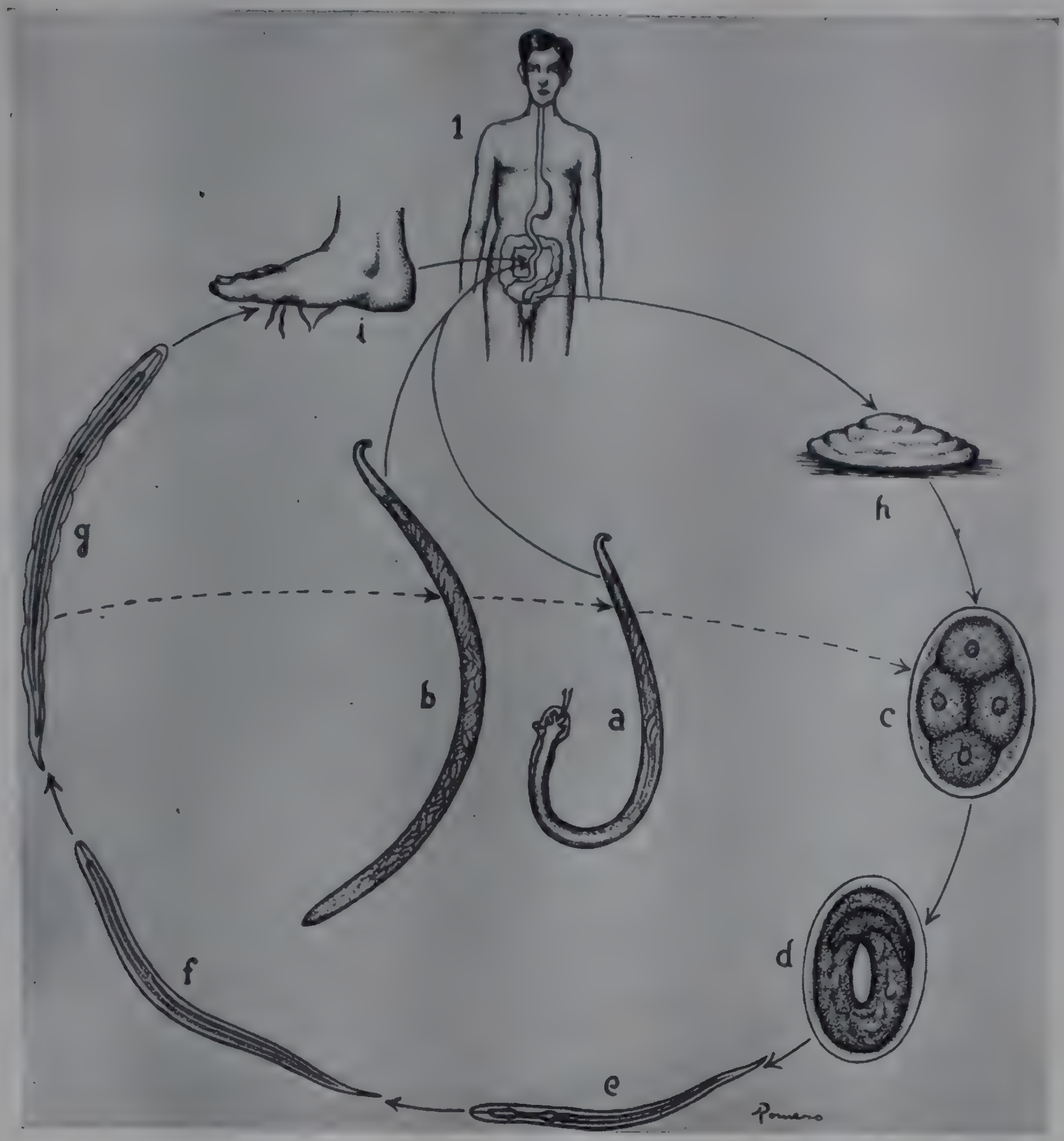


Fig. 510.—*Necator americanus.* Life cycle. *a* and *b,* Male and female adult parasites the habitat of which is the duodenum and jejunum. The eggs are passed in the feces (*h*); develop (*c* and *d*); a rhabditiform larva (*e*) is set free; it molts and becomes a filariform larva (*f*) which molts and becomes surrounded by a sheath; this is the infective larva (*g*) which bores through the skin (*i*), migrates through the heart and lungs, and reaches the intestines where it grows to the adult stage (*a* and *b*) after molting twice.

Extra-human stages (free-living): egg and three larval forms. Intra-human stages: two larval forms and the adult parasite.

(Original drawing of Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

CREEPING ERUPTION (LARVA MIGRANS)

The larvae of certain animal nematodes may occasionally bore through the human skin and live for a period of time in the dermis where they creep or advance, giving rise to an extremely pruriginous dermatitis along that part of the surface of the skin which is covered by the larva in its trajectory. This condition is known as *larva migrans* or creeping eruption. The original dermatitis may be complicated with pyodermatitis and superficial ulcerations following violent scratching.

Infection occurs on beaches when the skin comes in contact with sand that has been contaminated by egg-infested canine fecal material. Most of the cases have been caused by *Ancylostoma braziliense,* but *A. caninum* and others have also been reported. See Fig. 511.

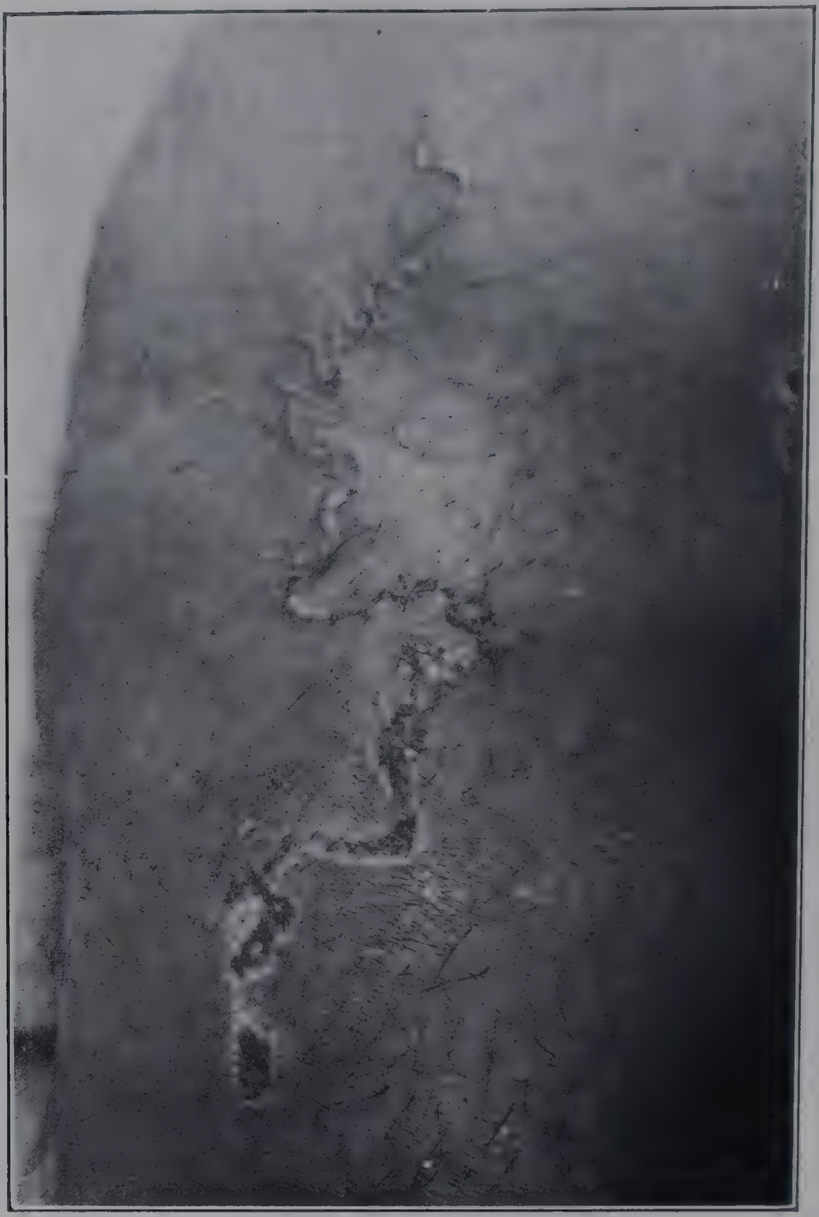


Fig. 511.—Clinical case of *larva migrans* studied by Dr. Kourí. Lesions on the internal surface of the right leg and knee.

Order **FILARIATA** Skrjabin 1915

The most important parasites in the order Filariata in human helminthology are the genera Wuchereria, Loa, Dirofilaria, Onchocerca, Dipetalonema or Ancanthoceilonema, and Mansonella filaria and microfilaria.

Family **FILARIIDAE** Clauss 1885

FILARIASIS BANCROFTI

Wuchereria Bancrofti (Cobbold 1877) da Silva Araujo 1877

Species in the genus Wuchereria are very delicate, with pointed anterior ends, and a broad head which is rounded and followed by a neck.

Filariasis bancrofti is caused by *Wuchereria bancrofti*. See Fig. 512. The adult worms are characterized by their extreme length and thinness, blunt extremities, and transparent opalescent white color. The male is 40 mm. long and 0.1 mm. wide; the female 80 to 100 mm. long and 0.3 mm. wide. The male parasite has a tendency to coil. The parasite is ovoviviparous.

The **eggs** are 40 microns long and 25 microns wide.

The females lay the embryos in the lymphatics. These embryos migrate to the blood stream and then invade the peripheral blood vessels while the host sleeps.

The embryo or **microfilaria** is approximately 300 microns long and 8 microns wide, and is enclosed in a sheath which is longer than itself. The caudal end is sharply pointed. In stained preparations, great numbers of embryonal nuclei or somatic cells can be seen. Where these somatic cells are absent, there are clear zones. In dried blood, the curvatures are regular, not sinuous.

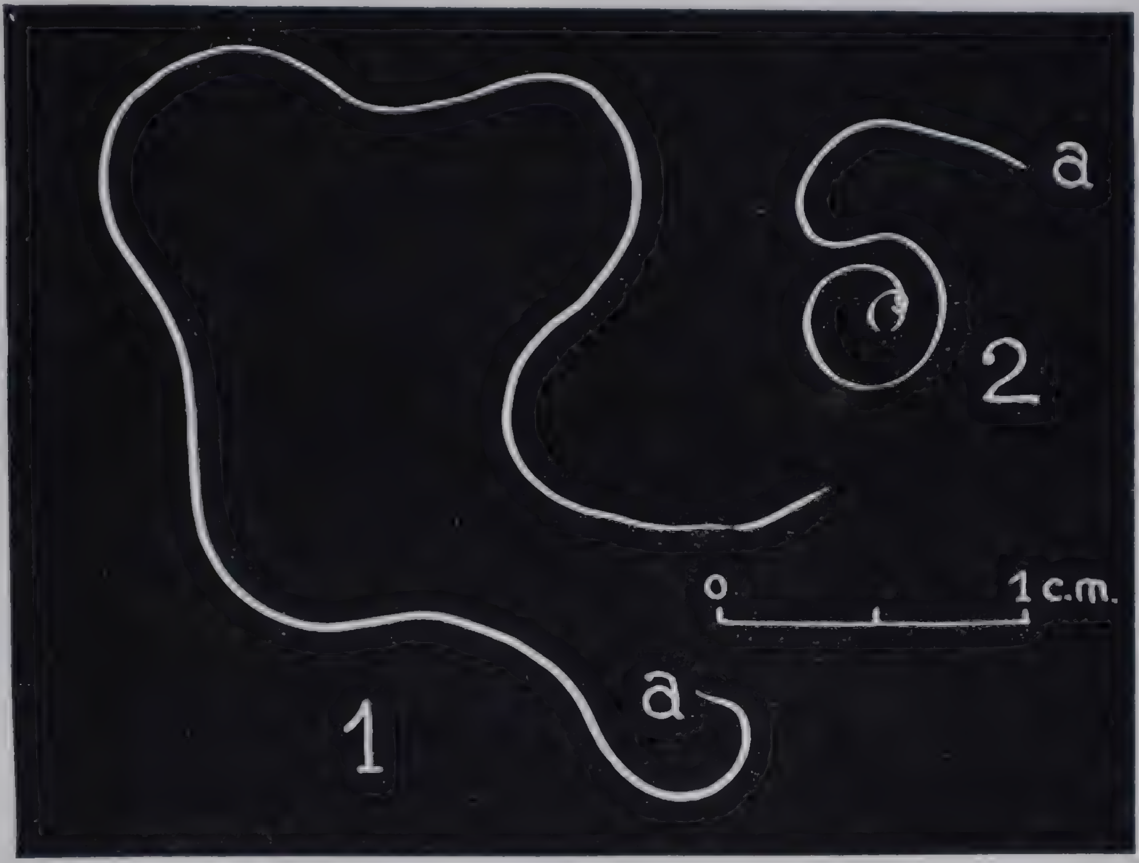


Fig. 512.—*Wuchereria bancrofti.* Two adults: female (*1*), 8.2 cm. long; and male (*2*), 3.7 cm. long. *a,* Anterior extremity. (Finlay Museum, specimen No. 1041.) The parasites, while alive, were tightly coiled; after several days in the refrigerator, where they died, they became uncoiled and took the form seen here after they were fixed in 10 per cent Formalin.

Life Cycle.—The life cycle is seen in Fig. 513.

The intermediate host is the *Culex fatigans.*

In Fig. 514 will be seen various types of lymphangitis, or elephantiasis, produced by this worm.

Diagnosis.—Parasites may be found in the blood often without any disturbances attributable to the parasite. Microfilariae are found in a drop of hemolyzed blood stained with Giemsa stain. Diagnosis is made by microscopic examination of a drop of blood obtained during the patient's sleep and examination of chylous fluid obtained any time during the day. The microfilariae appear in the peripheral blood at night and disappear almost completely during the day.

A good, rapid diagnostic method is to put defibrinated blood on a slide, allow it to dry, hemolyze it, and stain with Giemsa stain.

When this direct examination gives negative results, concentration methods are used. These methods consist in centrifuging a rather large volume of citrated blood, hemolyzing with distilled water slightly acidified by acetic acid, washing by centrifuging several times until the supernatant fluid is colorless, and then removing some of the sediment and examining it microscopically.

The **serologic test** recommended by Pandit and Iyer consists in mixing equal volumes of the serum of the patient and a suspension of microfilariae in citrated blood, and examining microscopically after 14 hours. In negative cases, agglutination of the microfilariae is observed; in positive cases, the leukocytes become fixed to the microfilariae, thus affecting their vitality. A large percentage of patients with elephantiasis give positive results with this test.

Biological Characteristics of Microfilariae.—

Microfilariae are from 100 to 400 times more numerous in the peripheral blood at night than during the day. During the day they accumulate in viscera, such as the capillaries of the lungs, left ventricle of the heart, cardiac muscle, aorta, and carotid arteries. They are never found in the liver, spleen, or bone marrow (Manson).

Rao has determined that the life span of microfilariae in the body of the host may be as long as 70 days. If inoculated into different unusual hosts, the microfilariae may live for variable periods.

TABLE 185.—KEY FOR IDENTIFYING THE MOST COMMON MICROFILARIAE IN HUMAN PARASITISM

1. Microfilariae in peripheral blood	3. Sheathed	3′.— Body nuclei or somatic cells do not extend to tip of tail; cephalic space equal to diameter of head; nocturnal periodicity (or non-periodic?)	*Wuchereria bancrofti*
		3″.— Nuclei extend in broken row to tip of tail; cephalic space twice diameter of head; nocturnal periodicity	*Wuchereria malayi*
		3‴.—Nuclei extend in continuous row to tip of tail; diurnal periodicity	*Loa loa*
	4. Unsheathed	4′.— Nuclei extend to tip of tail; frequently in a double row	*Acanthocheilonema perstans*
		4″.— Nuclei do not extend to tip of tail	*Mansonella ozzardi*
2. Microfilariae of skin and subcutaneous tissues		2′.— Nuclei do not extend to tip of tail; head enlarged; curved tail	*Onchocerca volvulus*
		2″.—Body nuclei or somatic cells extend to tip of tail; curved tail	*Microfilaria streptocerca*

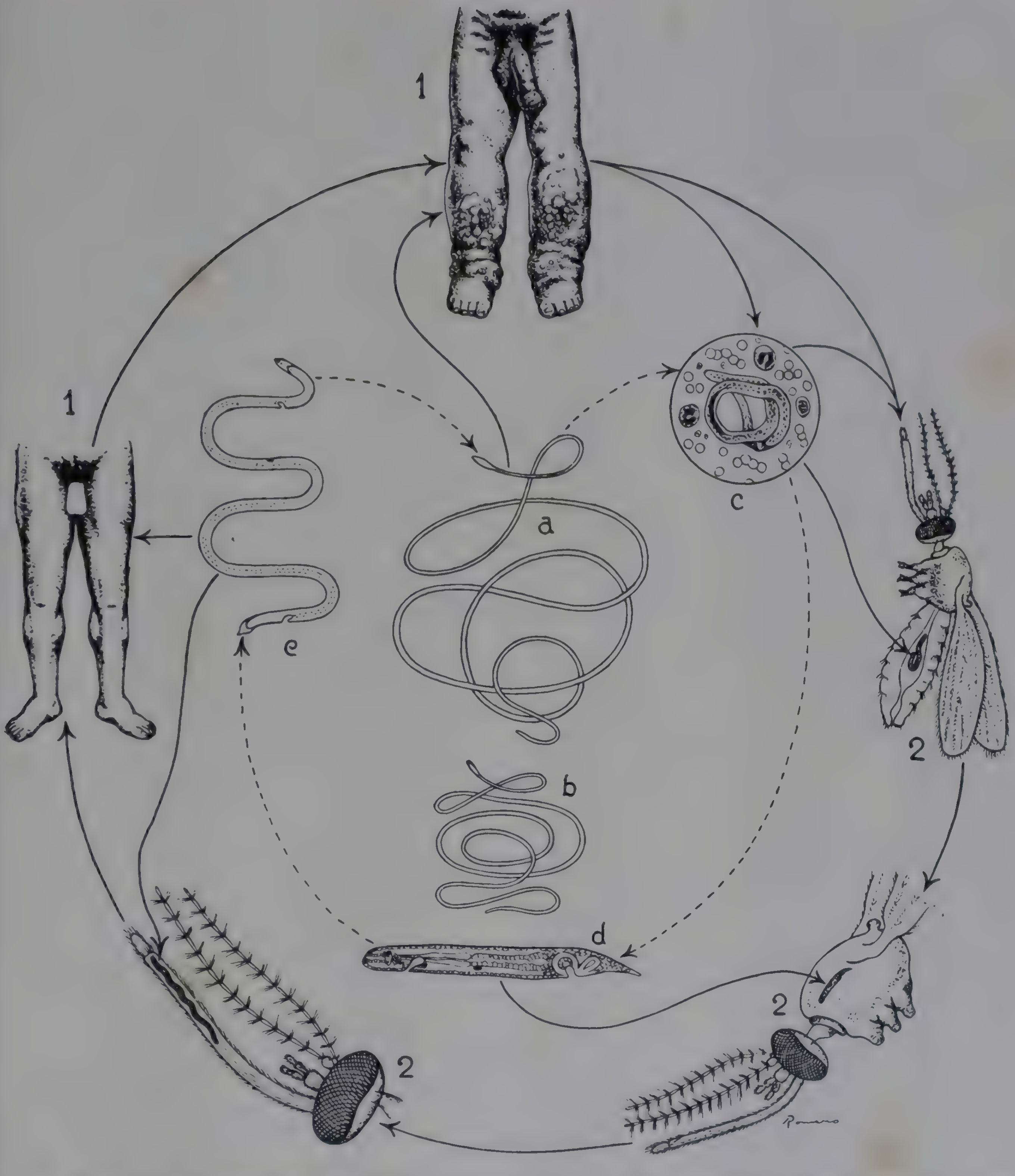


Fig. 513.—*Wuchereria bancrofti.* Life cycle. The adult parasites, *a* and *b*, live in the lymphatic trunks of their definitive host, man *1.* The female *a* deposits the embryos *c*, which are found in the peripheral blood usually while the parasitized patient is asleep, where they are extracted by the female mosquito *2.* They enter the stomach of the insect, lose their sheaths, and migrate toward the thorax, where they undergo two metamorphoses, first and second stages, *d*, and later another metamorphosis, to produce the infestive form *e* which enters the general cavity and the sheath of the proboscis of the mosquito. The mosquito bites the definitive host and the infestive forms *e*, boring actively through the skin, enter the lymphatic trunks where they grow into the male and female adult forms *a* and *b*, to begin a new cycle. (*a* and *b* modified by Manson; *d*, after Faust; *e*, from Belding; others after Kourí: Lecciones de Parasitología y Medicina Tropical, by Kourí and Basnuevo.)

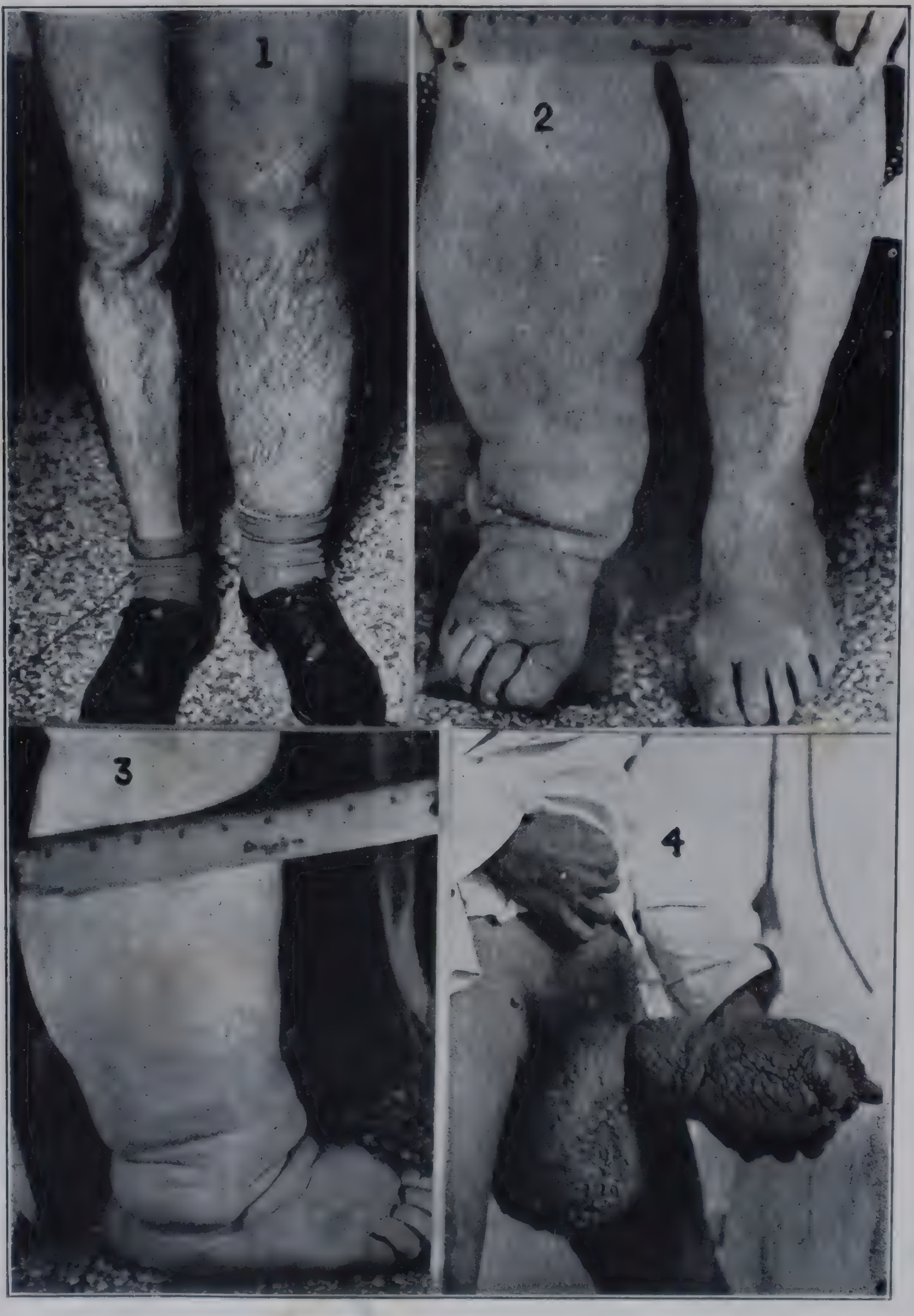


Fig. 514.—Wuchereriasis bancrofti—symptomatology: photographs of case of elephantiasis. *1*, *2*, and *3*, Various stages of elephantiasis of the leg (cases treated by Kourí). *4*. Elephantiasis of the penis and scrotum (case treated by Prof. Rafael Menocal).

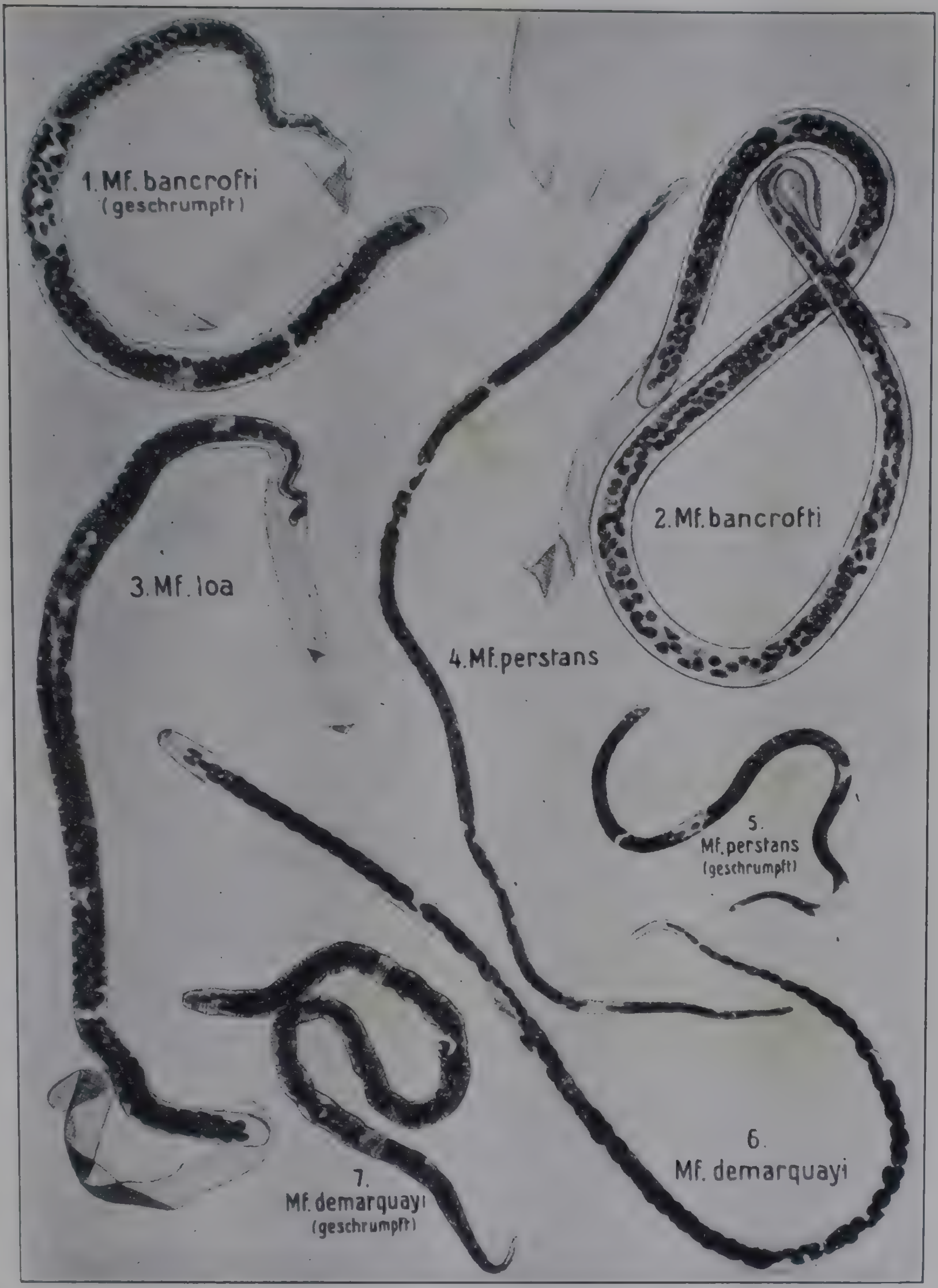

1. Mf. bancrofti
(geschrumpft)
2. Mf. bancrofti
3. Mf. loa
4. Mf. perstans
5. Mf. perstans
(geschrumpft)
6. Mf. demarquayi
7. Mf. demarquayi
(geschrumpft)

Fig. 515.—Important microfilariae of man (hematoxylin stain of thick drop preparations). Note certain changes in morphology due to shrinkage. *1, Wuchereria bancrofti; 2, Wuchereria bancrofti; 3, Loa loa; 4, Acanthocheilonema perstans; 5, Acanthocheilonema perstans* (shrunken); *6, Mansonella ozzardi; 7, Mansonella ozzardi* (shrunken). (After Fülleborn: Krankheiten und Hygiene der warmen Länder, Ruge-Mühlens-Zur Verth., Georg Thieme, Leipzig.

MALAYAN FILARIASIS

Wuchereria Malayi (Brug 1927) Rao and Maplestone 1940

The adult worm is similar to *W. bancrofti.* The female is smaller, being 55 mm. long and 0.16 mm. wide; the male is 23 mm. long and 0.09 mm. wide. It differs from *W. bancrofti* in minor details. The posterior extremity is twisted like a spiral.

The sheathed microfilaria is 177 to 260 microns long by 5 to 6 microns wide. It has a smooth cuticle, a double stylet at the anterior end, and two nuclei in the caudal extremity. The tail presents nuclei. Its nocturnal periodicity is less absolute than that of *W. bancrofti.*

Life Cycle.—Man is the only definitive host. The intermediate hosts are mosquitoes belonging to Mansonia, Anopheles, and Armigeres genera. The microfilaria undergoes two moltings in the mosquito and is transformed into the infective larva in 6 to 12 days at 29° to 32° C.

Diagnosis is made by finding the microfilaria in the peripheral blood.

LOAIASIS

Loaiasis is parasitism produced by *Loa loa.* This is a parasite of tropical Africa, particularly throughout a large part of the West African coastal region.

Loa Loa (Guyot 1778) Stiles 1905

The adult parasite is a white, opalescent, transparent organism attenuated at the two extremities, which are obtuse. It is a parasite of man exclusively. The male is 3 cm. long and 350 microns wide. The female is 5.5 cm. by 245 microns. They are ovoviviparous. Uterine eggs are 50 microns by 25 microns.

The larval form develops in various gadflies that act as intermediate vector hosts, especially the Chrysops.

The adult parasites localize in the subcutaneous cellular tissues of man, and the embryo in the blood. The **embryo or microfilaria** is 250 to 300 microns long and 6 to 8 microns wide, and is surrounded by a sheath. The sheath is much shorter than that of the embryo of *W. bancrofti,* and is difficult to stain. In stained preparations, the embryo shows a series of undulations. The somatic nuclei are larger than those seen in microfilariae of *W. bancrofti.*

Individuals after a night's sleep show microfilariae in their peripheral blood from 9 o'clock on in the morning; these increase in number up to 1 to 2 o'clock in the afternoon, and disappear toward 8 or 10 o'clock at night.

Life Cycle.—Mating doubtless takes place in the cellular subcutaneous tissue. The embryo develops in the uterus of the fertilized female, distending the membrane of the egg to form a sheath which protects the embryo, and in this form leaves the female, remaining free in the lymph. It then enters the blood stream, from which it is extracted by the intermediate hosts, which bite during the day when the embryos are abundant in the peripheral blood. After about 10 or 12 days, the infective larvae which have developed in the Chrysops accumulate in the inferior labium and leave at the moment of the bite, bore through the skin of the host in 60 seconds, and enter the subcutaneous cellular tissue, where they grow to adult stage.

Diagnosis.—The clinical diagnosis is confirmed by microscopic examination of blood, although at times the microfilariae cannot be found. There is frequently marked eosinophilia. The microfilariae have been found in the urine and saliva of patients.

ONCHOCERCIASIS

Onchocerca Volvulus (Leuckart 1893) Railliet and Henry 1910

Onchocerciasis is parasitism produced by *Onchocerca volvulus*. This disease is widely dispersed throughout Africa, but is more isolated in America. In America, it is found along the slopes of the cliffs overlooking the Pacific Ocean in Guatemala, and in Mexico in the States of Oaxaca, Chiapas, and Guerrero.

The American species localizes chiefly in the scalp, while the African species is commonly found in the skin of the trunk, especially in the region of the hips.

The male is 3 cm. long by 130 microns wide. The female can attain a length of 50 cm. and a width of 360 microns. The caudal extremity is curved backward and bears three papillae on each side of the anus.

The disease is characterized by the appearance of fibrous subcutaneous nodules located in the aponeurosis, in the muscular tissues, and in the periosteum of the cranium. These vary in size from pinhead to a hen egg. Each nodule harbors 4 to 5 males and 1 to 3 females interlaced with one another. These tumors, in the scalp, vary in number from 1 to 17. Patients show cutaneous, visual, and auditory disturbances.

DRACUNCULIASIS

Dracunculus Medinensis (Linnaeus 1758)

Dracunculiasis is parasitism produced by *Dracunculus medinensis*. It is found in Africa, in the valley of the Nile, in Equatorial Africa, and along the west and northwest coast. In Asia, it is found in Arabia, Persia, Afghanistan, Turkestan, and India. In 10 human cases found in the United States, the patients were foreigners, or the diagnoses were erroneous. It has been introduced into the Antilles, Guianas, and the District of Bahía in Brazil.

The gravid female is 50 to 1,200 mm. long by 0.9 to 1.7 mm. wide. The male, which is rarely found, is 12 to 29 mm. long by 0.4 mm. wide. It shows a cephalic prominence and a small oral aperture surrounded by an internal ring of 4 to 6 well-developed papillae.

Life Cycle.—Fertilization probably occurs in the beginning stages in the deep tissues of the host. The larvae, freed in the water, are ingested by appropriate species of Cyclops, and grow into the infective forms in about 3 weeks. The definitive hosts are infected by drinking water containing infected Cyclops. Other definitive hosts, in addition to man, are dogs, cats, wolves, and foxes. When the definitive hosts drink water infested with Cyclops, the larvae bore through the wall of the digestive tract and migrate toward the loose connective tissues.

Diagnosis.—Diagnosis is made by examining the local lesion for worms or larvae. Dead or calcified parasites may be located by x-ray.

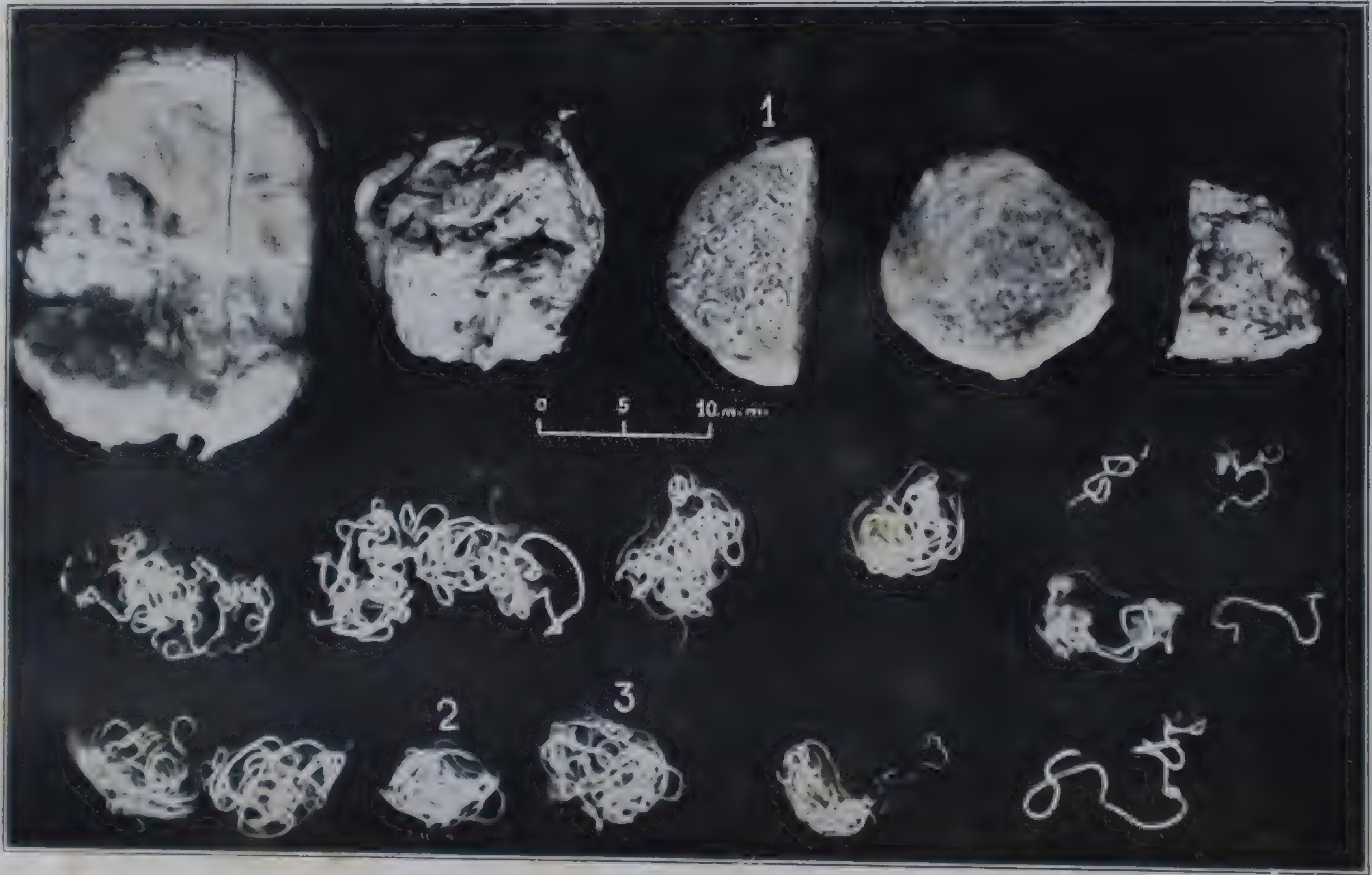


Fig. 516.—*Onchocerca caecutiens.* *Upper row*: several tumors produced by the parasite. *1*, represents a cross section of a fragment of tumor produced by *Onchocerca caecutiens.* It is enlarged in Fig. 518. *Lower rows*: bundles of parasites, obtained by artificial digestion of the tumors. *2* and *3* are enlarged in Fig. 517. (Original photograph of Kourí of material from Mexico sent by Dr. Eduardo Caballero.)

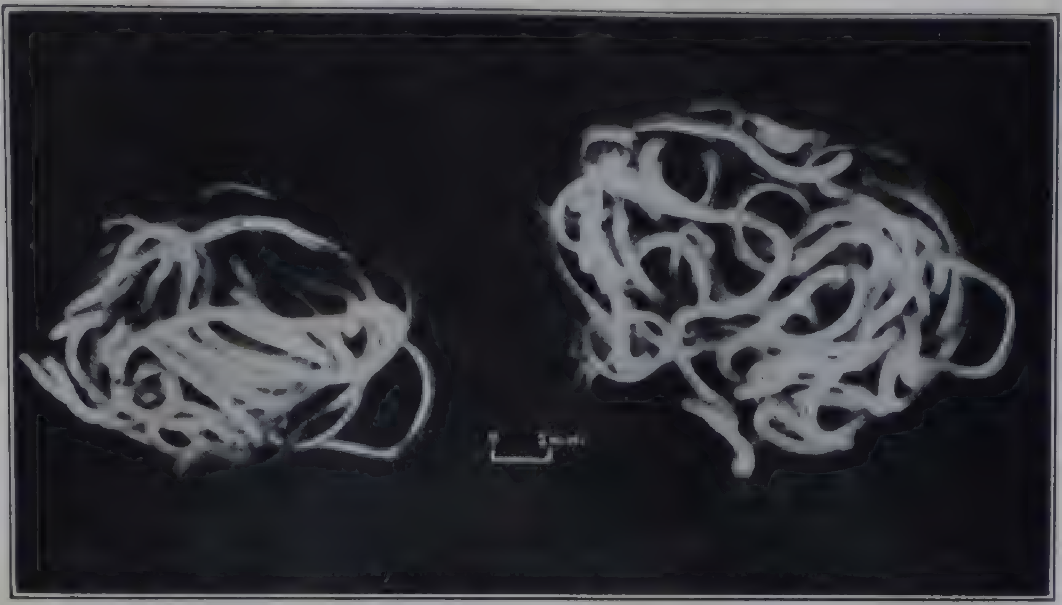


Fig. 517.—*Onchocerca caecutiens.* Enlargement of the bundles of *Onchocerca caecutiens, 2* and *3* in Fig. 516. (Original photograph of Kourí.)

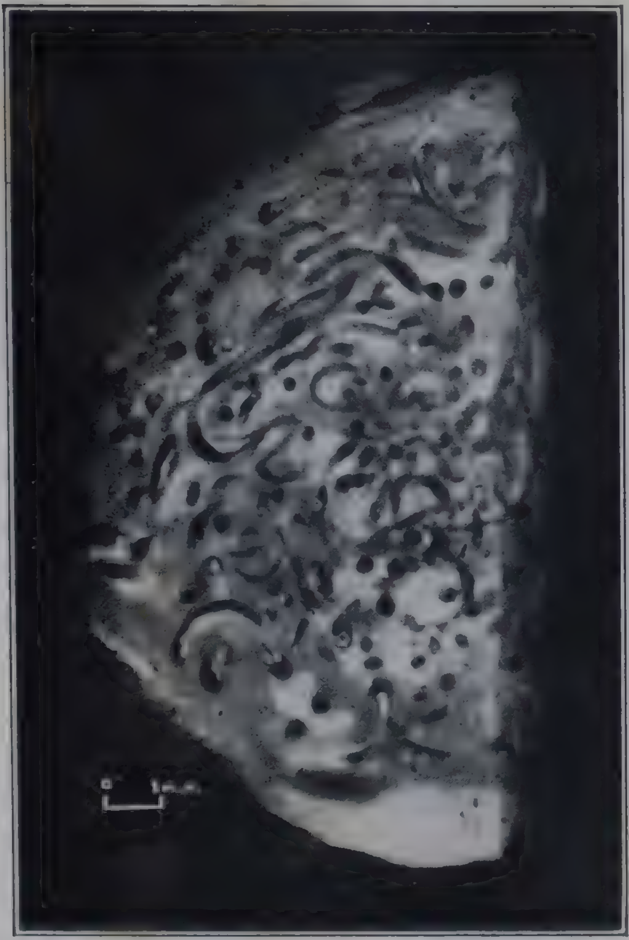


Fig. 518.

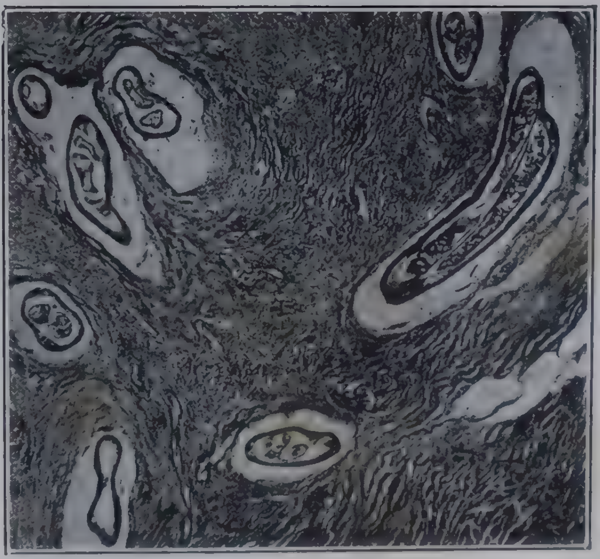


Fig. 519.

Fig. 518.—*Onchocerca caecutiens.* Enlargement of the tumor, *1* in Fig. 516. Section surface of one of the tumors. Note the cavities containing the parasites. Tumor fixed in 10 per cent Formalin. (Original photograph of Kourí.)

Fig. 519.—*Onchocerca caecutiens.* Histologic section of a fragment of the tumor in Fig. 518. Hematoxylin-eosin stain. Note the fibrous tissue filled with cavities containing the adult parasites. (Original photomicrograph of Kourí.)

MICROSCOPIC PSEUDOPARASITES IN NEMATODOLOGY

Under this heading are included all those microscopic elements which are found in secretions or excretions of man and which, through lack of knowledge or by erroneous interpretation on the part of technical workers, are confused with parasitic elements. Two large groups of microscopic pseudoparasites will be considered: (1) elements in transit; and (2) elements derived from outside contamination.

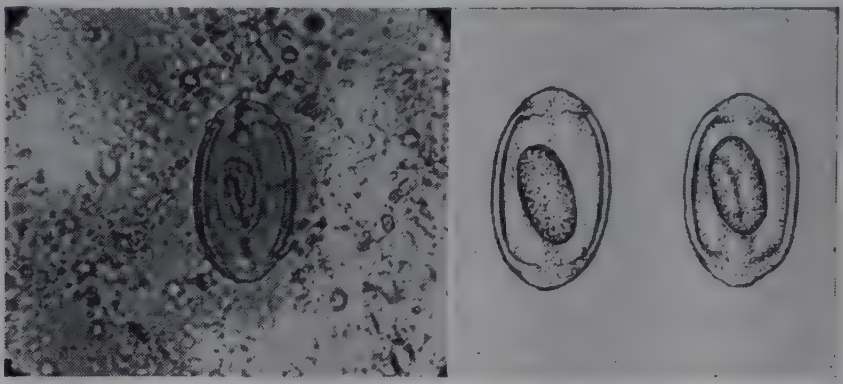


Fig. 520.—Microscopic pseudoparasites, original figure. Eggs in transit, found in human feces concentrated by Telemann method.

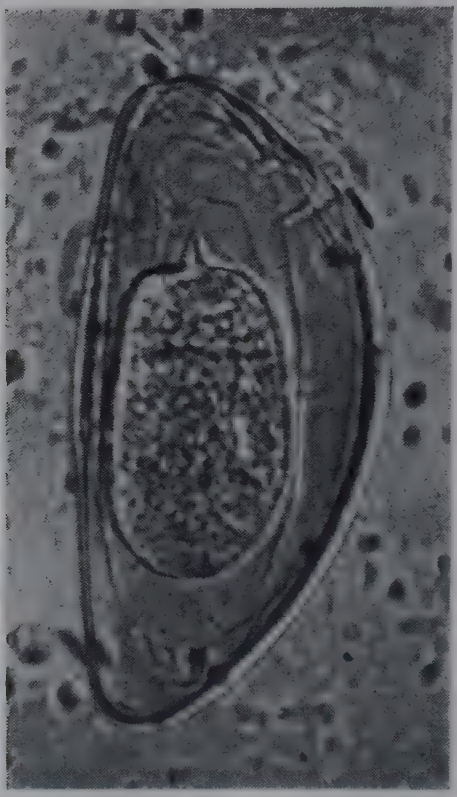


Fig. 521.—Microscopic pseudoparasites, original figure. Eggs found in the human feces concentrated by the flotation method using sugar cane and glycerine.

(1) **Elements in Transit.**—Included in this group are all those microscopic pseudoparasites found in the feces, which are simply elements in transit through the alimentary tract. These elements have been ingested in food and either undergo mild modifications or no modifications at all, and are eliminated in the excreta. In this group there are two categories: (a) eggs, larvae, etc., of Nemathelminthes of animals or vegetables which have been swallowed by man in animal or vegetable food and which are merely passing through the alimentary

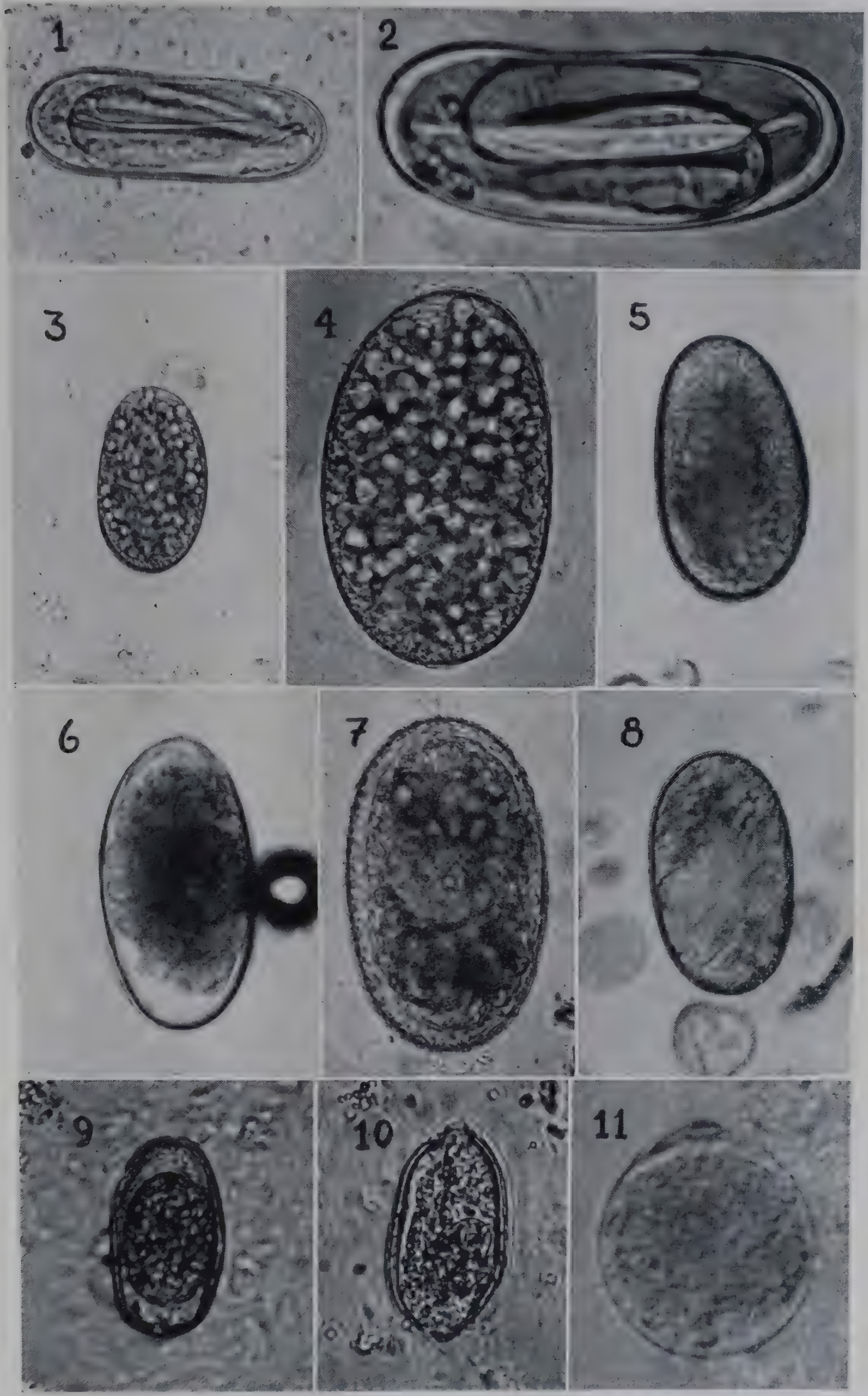


Fig. 522.—Microscopic pseudoparasites. Eggs in transit and nonparasitic elements, found frequently upon microscopic examination of human feces—microscopic pseudoparasites. Original figure. *1* and *2*, Egg of *Heterodera radicicola;* *3* to *8*, eggs of *Tyroglyphus* sp. in different stages of development; *9* and *10*, vegetable elements sometimes confused with eggs of helminths or with Coccidia; *11*, pine pollen grains, which frequently contaminate the stools.

tract without causing parasitism; and (b) elements not of parasitic nature, such as vegetable cells, pollen grains, etc., which are swallowed with food and may be mistaken for the eggs.

(2) **Elements Derived From Outside Contamination.**—This group also contains the two classes (a) and (b) described under (1) above. However, these are microscopic elements which have contaminated the stools after they were passed, either because the stools were collected in dirty containers or because the elements dropped into the specimen in the container.

PHYLUM PLATYHELMINTHES

(Flatworms)

Platyhelminthes, or flatworms, are metazoa with soft integument; parasitic adult forms without body covering; bilaterally symmetrical; lacking coelomic cavity; usually flat, more rarely cylindrical (Schistosoma). The majority are hermaphroditic, although some have separate sexes (Schistosoma). They are almost always tape-like and segmented (Cestoda), or leaf-shaped and not segmented (Trematoda); without digestive tract (Cestoda) or provided with an incomplete digestive tract, without anus (Trematoda); excretory system bilaterally symmetrical, ending in flame cells or "solenocytes." The Platyhelminthes generally have organs of attachment, such as suckers and hooklets. The majority are endoparasites.

The Cestoidea are exclusively parasitic; adults are hermaphroditic, covered with a nonciliated integument. When present, the ciliated epithelia are confined to the embryo which emerges from the egg (coracidium). Scolex is provided with suckers and frequently with hooklets which enable the worms to attach themselves to the tissues of the host. No digestive tract. In most of the species, the bodies are divided, transversely, into sexually complete, separable entities, called proglottids.

Order **CYCLOPHYLLIDEA** Braun 1900

Family **TENIIDAE** Ludwig 1886

TAENIASIS

Taenia Saginata Goeze (1782)

Taeniasis saginata is parasitism produced by *Taenia saginata,* the beef tapeworm. It is found in almost all of the countries of the world except the Slavic countries Czechoslovakia and Yugoslavia. It is acquired by eating inadequately cooked infected beef. It is found more frequently than the pork tapeworm, *T. solium,* which is common wherever raw or inadequately cooked or processed pork is consumed by man.

T. saginata is 4 to 10 meters long. Three portions are important: the head or **scolex,** the **neck,** and the body trunk or **strobila,** which is composed of segments or **proglottids.** The shape of the scolex, when viewed with a hand lens or under the low power of a microscope, is fusiform, with a terminal quadrangular base. It has two pairs of elliptical suckers, 0.7 to 0.8 mm. in diameter, which usually contain a black pigment. It lacks a rostellum and has no hooklets. A neck, which is smooth, connects the scolex with the strobila.

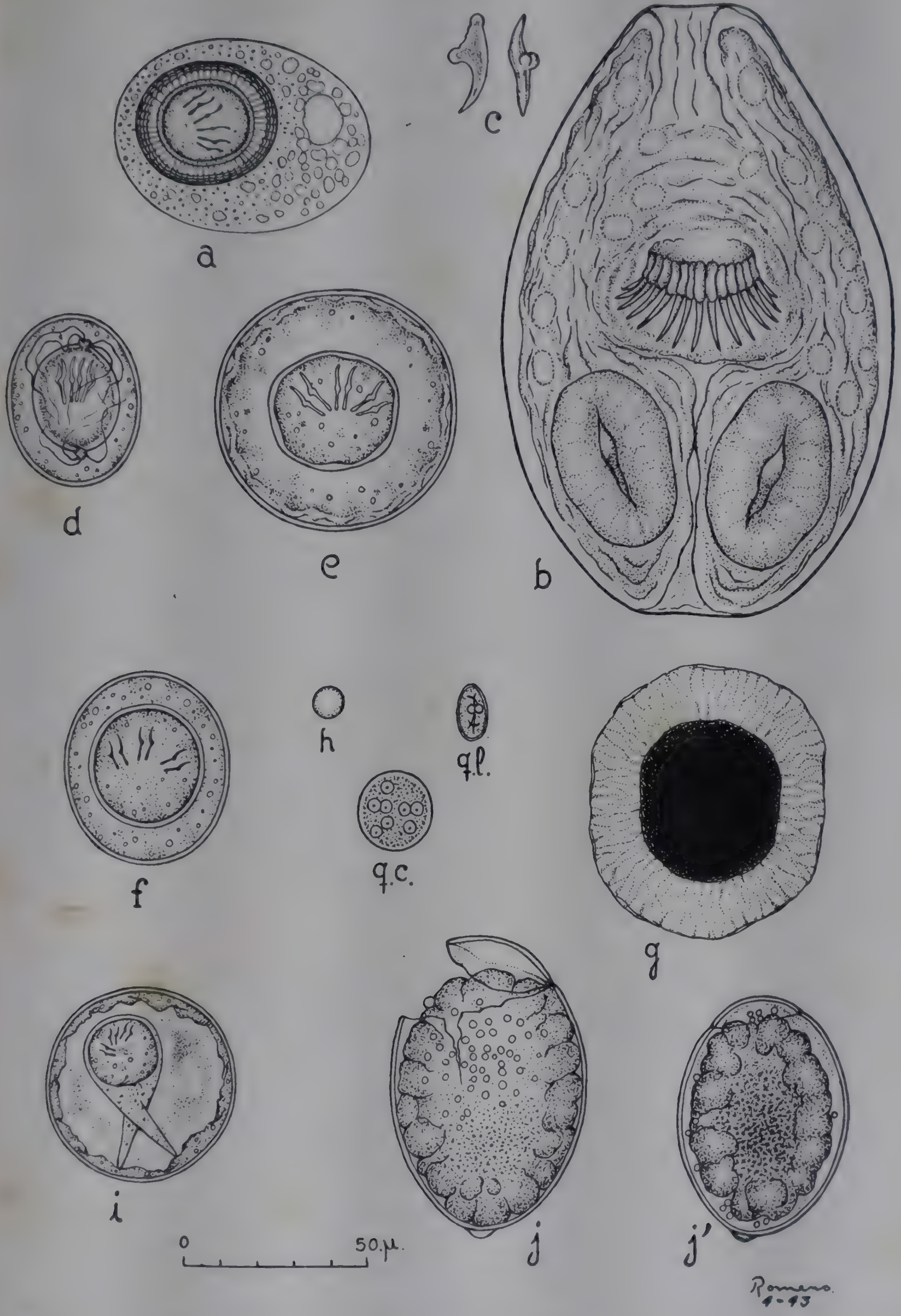


Fig. 523.—Cestodes. Eggs, oviferous capsules, scolex, and hooklets. Original drawing, at the same scale, with camera lucida. *a*, Eggs of *Taenia saginata;* *b*, scolex of the larva of *Echinococcus granulosus* (hydatid sand) fixed in 10 per cent Formalin; *c*, hooklets; *d*, eggs of *Hymenolepis nana;* *e*, eggs of *Hymenolepis diminuta.* *f*, Egg of *Dipylidium caninum.* *g*, Oviferous capsule of *Inermicapsifer cubensis.* *i*, Egg of Anoplocephalinae of the hutia conga. *j,j'*, Eggs of *Diphyllobothrium latum.* (*j*) crushed to show the opened operculum. Human erythrocyte (*h*), cyst of *Giardia lamblia* (*q.l.*), and cyst of *Endamoeba coli* (*q.c.*) for comparative purposes. (After Kourí in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

The gravid proglottid, outside the body of the host, contracts and expels a white powdery-appearing substance, which is composed of thousands of eggs (Bacigalupo phenomenon).

Eggs.—The eggs consist of an ovoid embryophore 20 to 30 microns wide and 30 to 40 microns long, with a thick, dark brown, radially striated wall. Within this embryophore is a hexacanth or 6-hooked embryo or onchosphere, 20 microns in diameter. The gravid proglottid tears open its terminal uterine branches when it separates from the strobila, permitting the eggs to escape.

Life Cycle.—The life cycle is given in Fig. 526.

When the patient eats raw or slightly cooked beef containing the larvae, these larvae, *Cysticercus bovis,* reach the intestine. Intestinal secretions digest the wall of the cyst, liberating the scolex. The scolex anchors itself to the mucosa, the neck proliferates to form the proglottids, and the adult worm develops. It takes two to three months to form the adult taenia. At the end of this period, the expulsion of gravid segments begins, and thus dissemination of the eggs onto the soil. If these eggs are then ingested by cattle, a new cycle begins.

Cysticercus Bovis.—*Cysticercus bovis,* the larval form of *T. saginata,* is an ovoid vesicle 5 to 10 mm. long and 3 to 4 mm. wide. In muscle tissue, it is surrounded by a fibrous capsule. It offers an elastic resistance when pressed between the fingers. The larva has two pairs of suckers and is unarmed as is the adult taenia. The larva dies at a temperature of 46° C., but remains alive for about 40 days in meat kept at 1° to 4° C. It is destroyed at −8° to −10° C. in 3 hours. Parasitism by the larval form is known as bovine cysticercus.

Taenia Solium Linnaeus 1758

Infestation by *T. solium* is known as taeniasis solium, the pork tapeworm disease. The adult tapeworm is 2 to 3 meters long, but may be as long as 8 meters or more. The globular scolex has a diameter of 600 microns to 1 mm. and is pigmented. The rostellum is apical, is capable of invaginating, and bears 2 crowns of hooklets arranged in alternate rows, numbering 22 to 32. They vary in size from 160 to 180 microns; some are smaller. The neck is short. Behind this structure are the immature, mature, and gravid proglottids. The fully developed proglottid is almost square. Gravid proglottids are not as long as those of *T. saginata.*

Eggs.—The eggs are expelled with fragments of the strobila after expulsion from the patient. They contain a hexacanth embryo or onchosphere about 20 microns in diameter and armed with 6 stylets. Eggs are indistinguishable from those of *T. saginata.*

Life Cycle.—Outside the human body the eggs are viable for weeks in soil. When ingested by hogs (or man), they hatch immediately. The liberated onchosphere penetrates the intestinal wall, reaches the lymphatic or circulatory system, and the embryos are distributed throughout the body. They usually localize in the musculature or subcutaneous tissue. Within 60 to 70 days they metamorphose into infective bladder worms or *Cysticercus cellulosae,* measuring 5 by 8 to 10 mm. Cysticerci reach man when he eats raw or inadequately prepared pork. The larvae are digested out of the cysts, become attached to the intestinal wall, and grow to maturity in 4 to 12 weeks.

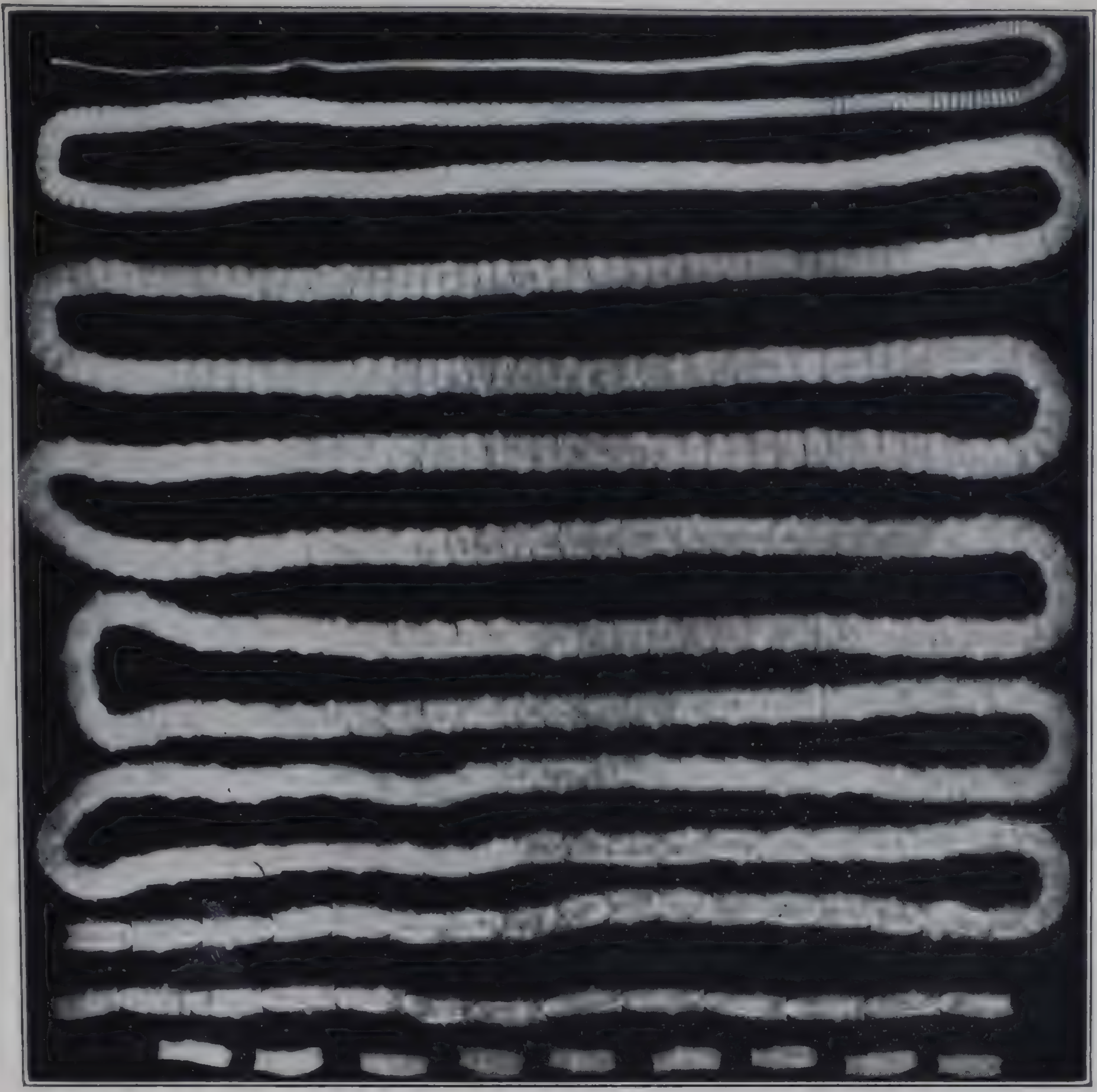


Fig. 524.—*Taenia saginata.* Complete specimen expelled after treatment with ether extract of male fern and carbon tetrachloride in oily solution. Note the scolex and fine portions of the strobila, above; and the gravid proglottids, separated from the strobila, below. (Original photograph of Kourí.)

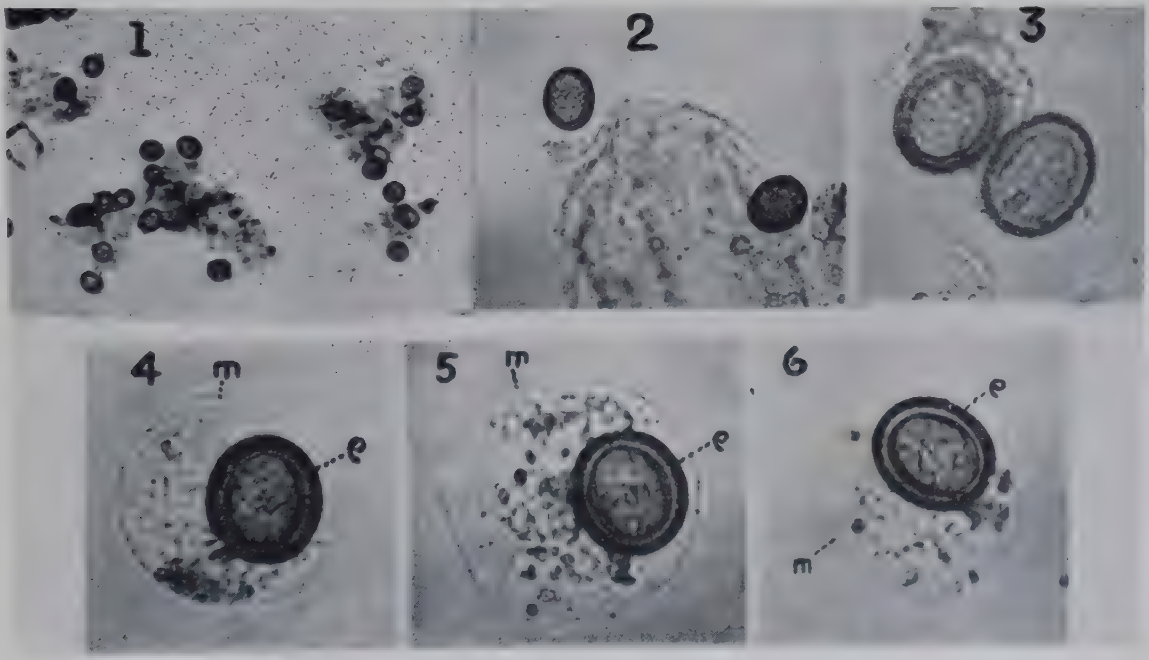


Fig. 525.—*Taenia saginata.* *1* to *6*, Photomicrographs of eggs, with progressively increasing magnification; *4*, *5*, and *6*: *m*, external membrane of the egg; *e*, embryophore containing onchosphere or six-hooked embryo. (Originals of Kourí.)

Man is the definitive host, the hog the intermediate host. Human infection results from ingestion of viable eggs.

Differential Characteristics of Taenia Saginata and Taenia Solium

The scolex of *T. saginata* is quadrangular and unarmed, that is, it bears no rostellum or hooklets, while the scolex of *T. solium* is spherical and smaller, and bears a rostellum with two crowns of hooklets. *T. saginata* is usually longer and contains a greater number of segments. *T. solium* is shorter, has a smaller number of segments, its genital pores are less regularly staggered, and the vagina is not provided with a sphincter. The larvae of *T. saginata (Cysticercus bovis)* localize in adipose tissue and striated muscles. The larvae of *T. solium (Cysticercus cellulosae)* localize in the muscle tissue of the pig, and accidentally in that of man.

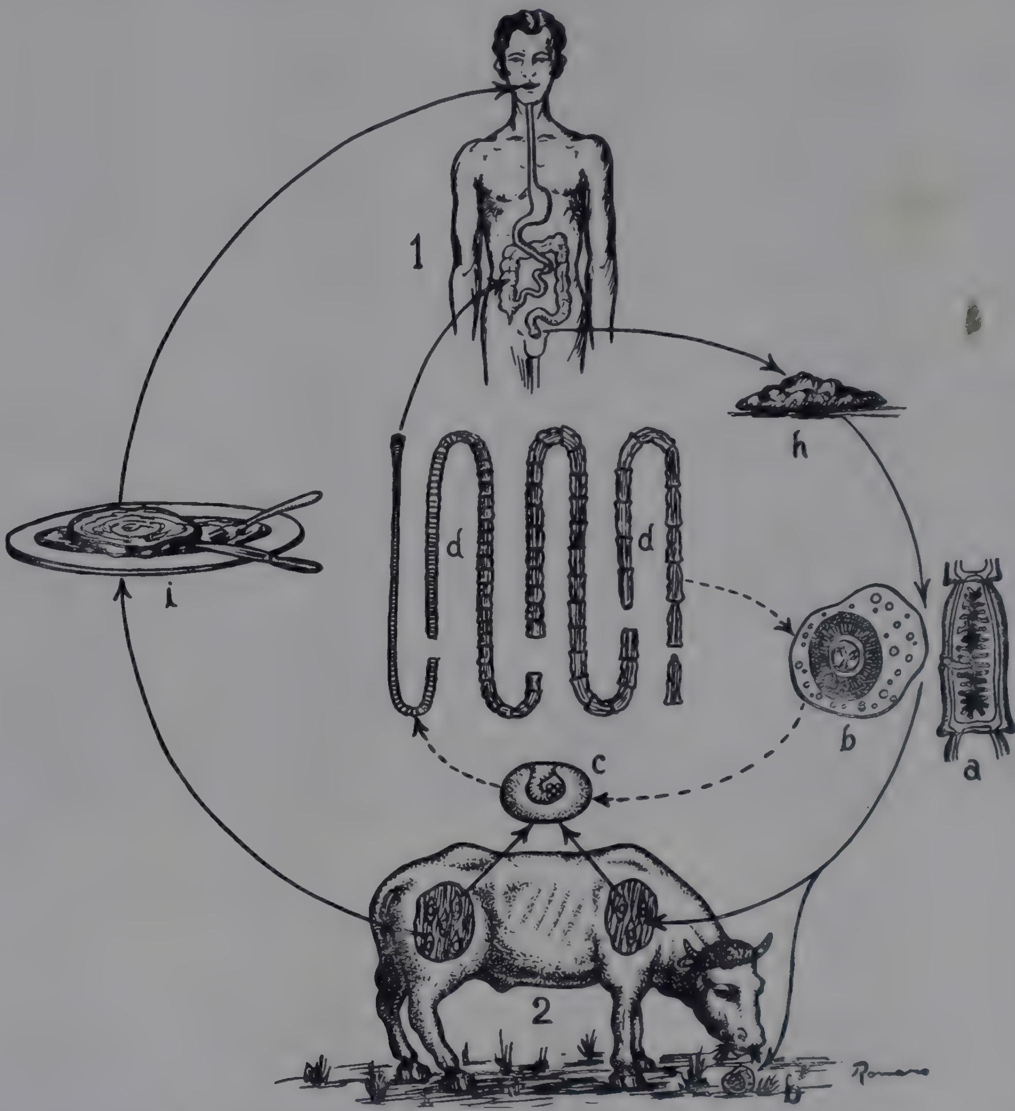


Fig. 526.—*Taenia saginata.* Life cycle. Man is the definitive host (*1*), harboring the adult parasite (*d, d*) in the small intestine. In the feces (*h*), gravid proglottids (*a*) and eggs (*b*) are passed and (*b'*) are ingested by cattle (*2*), the intermediate host. *Cysticercus bovis* (*c*), which is the larval stage, develops in the muscular system of cattle. This Cysticercus develops into the adult Taenia (*d, d*) in the small intestine of man (*1*) when ingested with raw or poorly cooked beef (*i*), beginning a new cycle. (Original drawing of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

Laboratory Diagnosis

The laboratory worker may have to deal with one of three contingencies: (1) The patient may bring him the segments of the parasite. (2) The patient may bring him a sample of the feces. (3) The patient may merely consult him without these objective findings.

(1) The segments are usually discovered by the patient himself in the feces, on his own body, or on his clothes. Segments are occasionally found in surgically removed appendices. Quite rarely they are vomited or found in abscesses or fistulae, umbilical or perineal. If the segments are brought into the laboratory wrapped in paper without water, or in gauze, they dry out, but when placed in water they resume their former typical appearance of gravid segments. By rubbing the dried proglottid lightly with a wet platinum loop, and placing the material thus obtained in a drop of water on a clean slide, a preparation is obtained which, when observed microscopically, will most likely show numerous eggs of the parasite. There is no difficulty when the fresh proglottids are brought for examination, dead or alive, in a small flask or test tube, in water, alcohol, or 10 per cent Formalin. In 10 per cent Formalin, they retain their original size and white color.

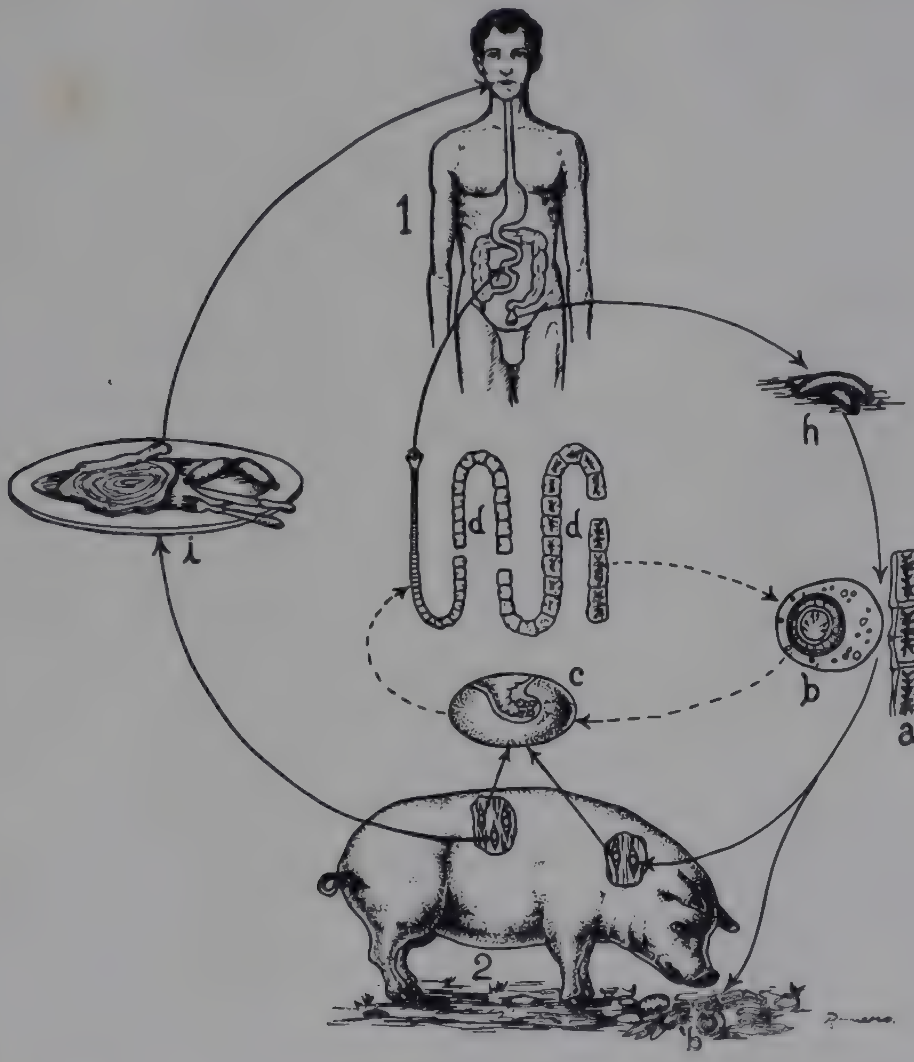


Fig. 527.—*Taenia solium.* Life cycle. This parasite follows the same cycle as *Taenia saginata* except that the intermediate hosts for *T. solium* are swine, which harbar the larval form, *Cysticercus cellulosae.* (Original drawing of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

(2) If the patient brings a sample of feces to the laboratory, the procedure is (a) immediate macroscopic examination; (b) microscopic examination; and (c) macroscopic examination by screening the feces.

Step (a) is very important, for, in the majority of cases, enough proof is obtained by macroscopic examination to establish an accurate diagnosis. The segments can be seen in the feces specimen in a wide-mouthed bottle.

Step (b), a complete microscopic examination of the stool, includes (1) a direct thick preparation; (2) a direct thin preparation; (3) a thin preparation stained with Lugol solution; (4) a preparation of feces concentrated by one of the low density solutions, such as Telemann's or others; (5) a preparation of feces concentrated by one of the high density solutions; (6) a preparation of a dilution of the stained and centrifuged feces.

All these slide preparations should be covered by cover glasses 22 mm. by 22 mm., and the entire specimen should be examined carefully.

Indirect Methods.—Eosinophilia is frequent though not constant or specific in parasitism by *T. saginata.* Complement fixation and intradermal reactions, applicable in cases of echinococcosis and other parasitoses, have not given satisfactory results in cases of parasitism by *T. saginata* and *T. solium.*

HYDATID DISEASE

Hydatid disease is produced by the larval form of *Echinococcus granulosus.*

Echinococcus Granulosus (Batsch 1786) Rudolphi 1805

E. granulosus, and its larval form, the hydatid cyst, are found on all the continents. The parasites live in any climate. Factors favoring infestation are occupation, personal habits, and food habits. In those countries where cattle raising is one of the principal industries, where meat and milk and all their derivatives, sausage, cheese, and butter, are important products, or wherever man and dog inhabit the same territory, conditions are ideal for the spread of this disease.

E. granulosus, in its adult form, is a habitual parasite of the intestines of dogs, jackals, and wolves. In its larval form, it parasitizes the tissues of man, pigs, sheep, cattle, etc., and forms what is known as ''hydatid cyst.''

This tape worm is the smallest of the important cestodes of man. It is 3 to 6 mm. long, 300 microns in diameter. It is provided with four ovoid suckers, has a projecting rostellum with a double crown of 30 to 36 hooklets. The neck is short, becoming narrow toward the back. The strobila is composed of 3 to 4 proglottids.

Eggs.—There are from 500 to 800 eggs in the gravid proglottid. They resemble those of *T. saginata* and *T. solium,* and are from 32 to 36 microns long and 21 to 30 microns wide. They contain a hexacanth embryo 25 microns long.

Larva or Hydatid Cyst.—The hydatid cyst is a tumor of variable size, which may reach the size of the head of an adult human being. It is frequently localized in the liver, lung, abdominal cavity, pelvis, or any other part of the host. It has an elastic consistency and is composed of two parts, a fibrous capsule, and the larva, which is found inside this fibrous capsule.

Brood Capsules and Scolex.—Brood capsules are formed by proliferation of the internal germinal layer. They are spherical; their walls have the same structure as that of the mother layer. This wall is very thin; it gives rise,

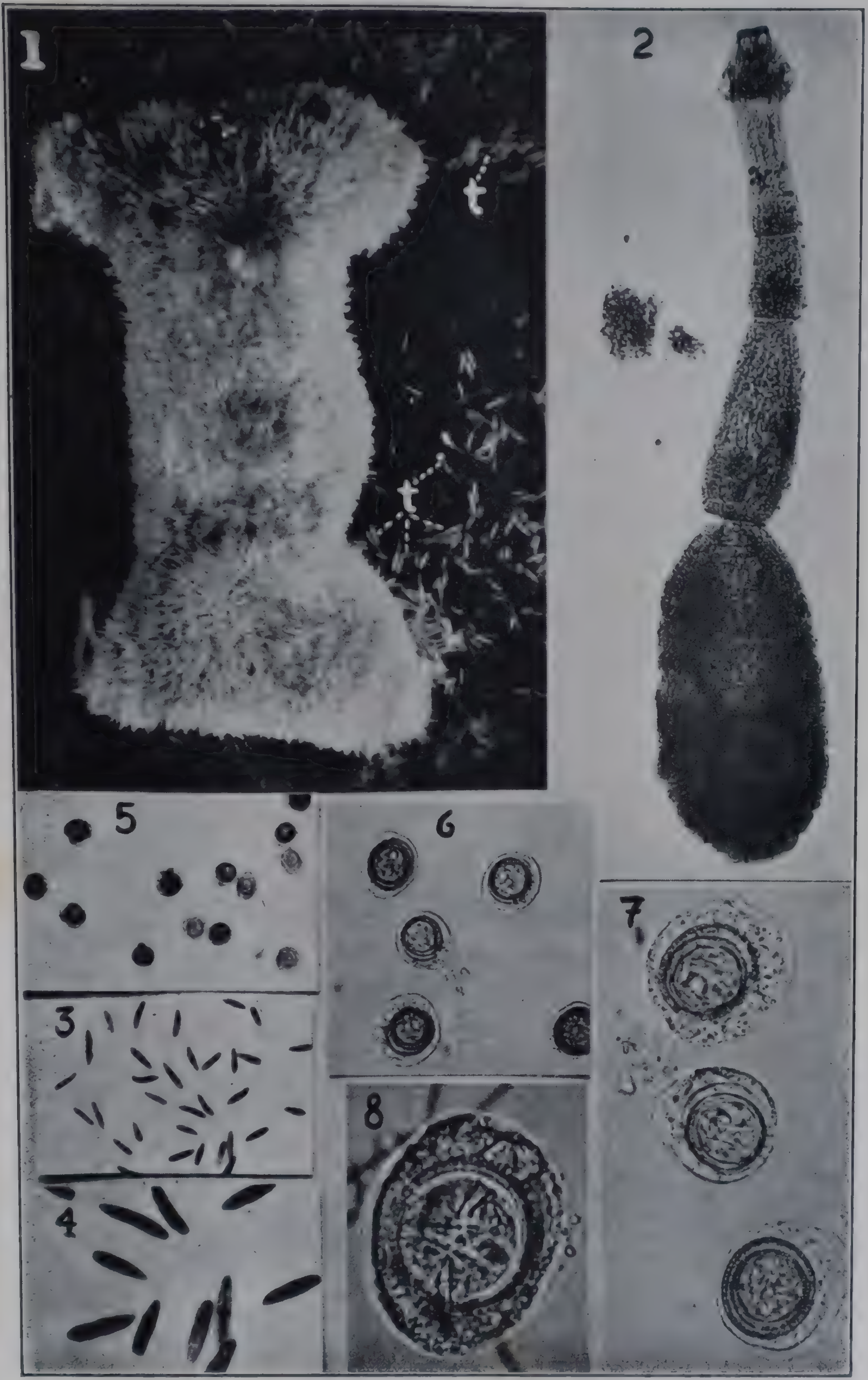


Fig. 528.—*Echinococcus granulosus*. *1*, Intestine of a dog experimentally infested with *E. granulosus* through ingestion of a hydatid cyst (Aguirre Pequeño). The intestine is filled with taenias, many unattached. *2*, Photomicrograph of adult taenia. *3*, Gravid segments, natural size. *4*, Same segments magnified. *5* to *8*, Eggs obtained from gravid segments, photomicrographs of different magnifications. (*3* to *8*, Original photographs by Kourí of material sent by Dr. Aguirre Pequeño from Mexico.)

through internal proliferation, to a variable number of scolices. These brood capsules, at first attached by a broad base, develop a pedicle, and finally are set free in the fluid of the cystic cavity. The scolices remain attached by a thin pedicle. When the brood capsule bursts open, the scolices become arranged in radial formation around the membrane which is now in the center.

Daughter Cysts or Vesicles.—The daughter cysts may be endogenous or exogenous, depending on whether they are found inside or outside the true wall of the mother cyst. The wall is the same as that of the mother cyst. There is a fluid in the cavity of these daughter cysts, with the same characteristics as the fluid in the mother cyst.

Hydatid Cyst of the Liver and Other Organs.—A cyst in the liver may never be noticed. When symptoms do appear, it is usually several years after the beginning of the disease. At first hepatic and epigastric pains are present. Lack of appetite, most frequently for fats, usually accompanies these symptoms. There is often a history of urticarial crisis, and a high percentage of eosinophiles in the blood. The tumor attains a voluminous size and pushes out the abdominal wall, causing a noticeable distention of the abdomen.

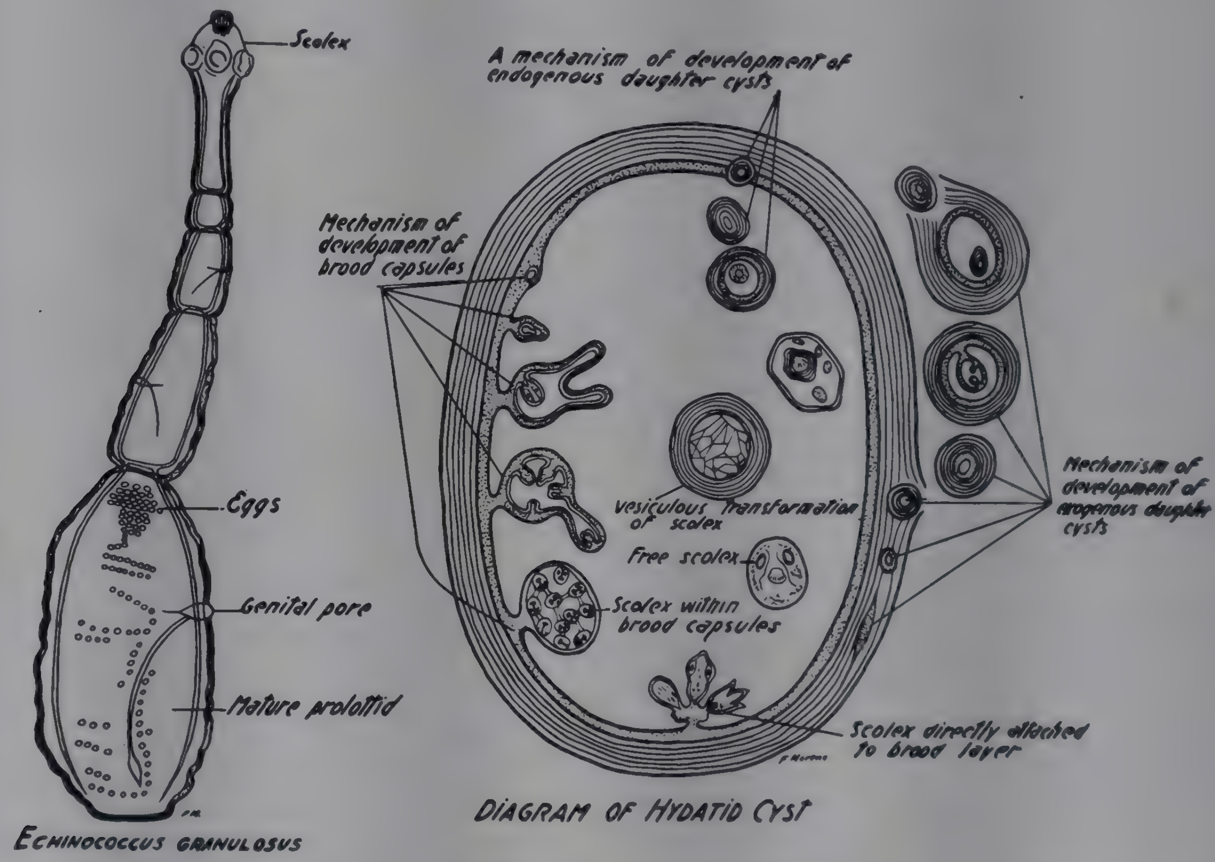

Scolex
A mechanism of development of endogenous daughter cysts
Mechanism of development of brood capsules
Eggs
Genital pore
Mature prolottid
vesiculous transformation of scolex
Free scolex
Scolex within brood capsules
Mechanism of development of exogenous daughter cysts
Scolex directly attached to brood layer
Echinococcus granulosus
Diagram of Hydatid Cyst

Fig. 529.—Schematic drawing of *E. granulosus.* Adult parasite, left, and larva in a hydatid cyst, right. (According to Leuckart in Blanchard and Kourí.)

There may be hydatid cyst of the lung, of the peritoneum or the pelvis, of the kidneys, the brain, bones, or circulatory system.

Cysts may become calcified and degenerated, and retract. Bacterial infection between the cuticular and adventitious membranes kills the larva. Bacteria enter the hydatid fluid, which, being a good culture medium, aids their growth; abscess formation follows.

Life Cycle.—The life cycle of this parasite is found in Fig. 531.

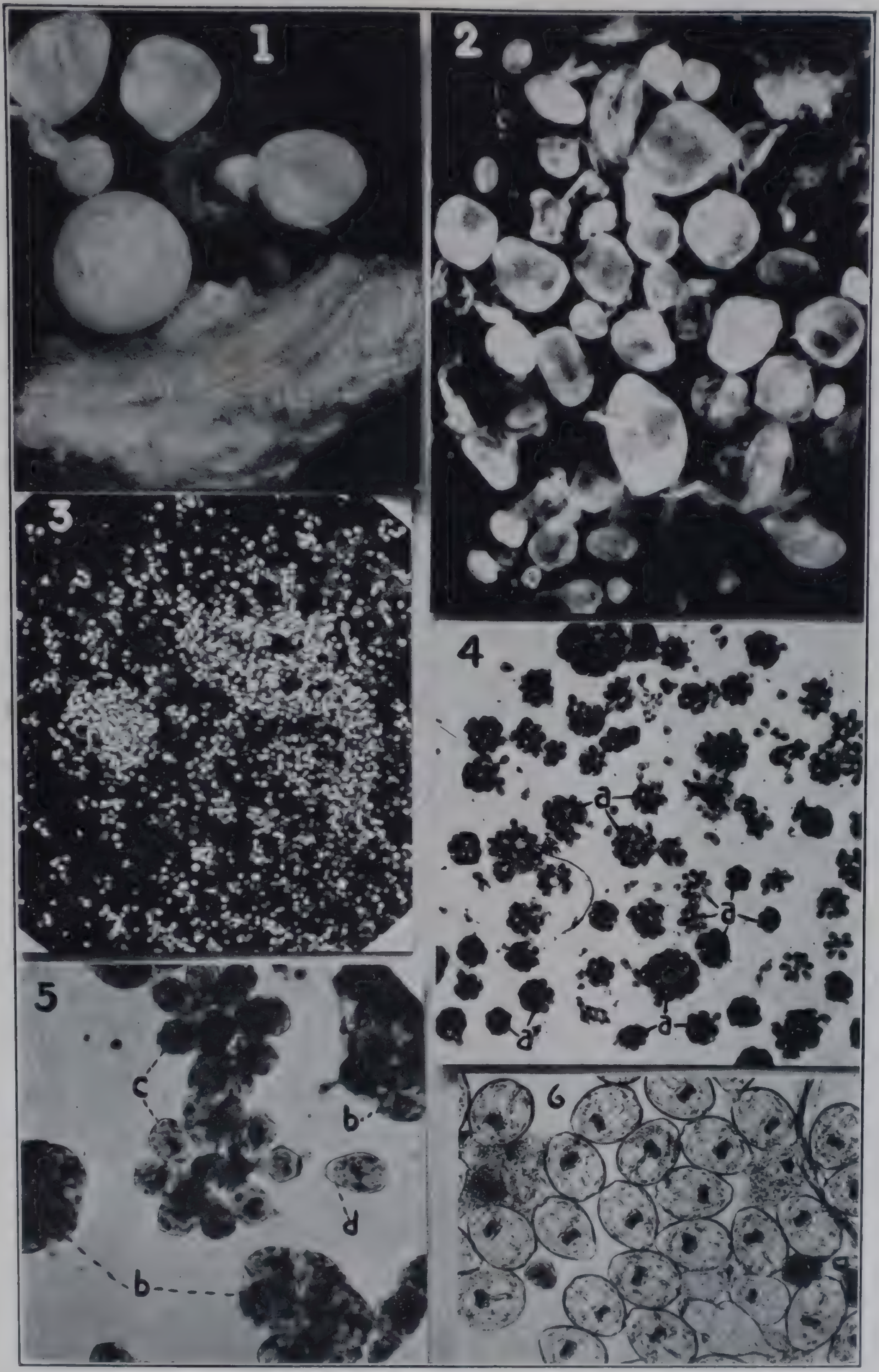


Fig. 530.—*Echinococcus granulosus.* (Original of Kourí.)

1 and *2*, Endogenous daughter cysts, natural size. Hydatid cyst of the liver opening into bile ducts. Cysts expelled in the feces. *2*, Case of Kourí.

3, Photograph, slightly enlarged: "hydatid sand." Material sent by Prof. Bacigalupo from Buenos Aires. Each grain of sand is a brood capsule enclosing a varying number of scolices. Dark background, illuminated from above.

4, Photomicrograph of "hydatid sand," low power. Each brood capsule *a* encloses a variable number of scolices and represents a grain of sand.

5, Brood capsules *b*, slightly magnified. Each consists of a group of scolices enveloped by a germinal membrane. This membrane, when broken, assumes a central position, with the scolices radially attached, *c*. *d*, free scolex.

6, Free scolices, low magnification.

Diagnosis.—Diagnosis may be made by finding hooklets, scolices, vesicles, membranes, or hydatid fluid in feces, bile, urine, sputum, or vomica, or in the material obtained by puncture, by operation, or at autopsy. The hooklet is important in diagnosis, because of its resistance to disintegration, even in degenerating or suppurating cysts.

In most cases, indirect serologic methods must be used to establish a clinical diagnosis. Precipitin, complement fixation, and intradermal reaction tests may be used.

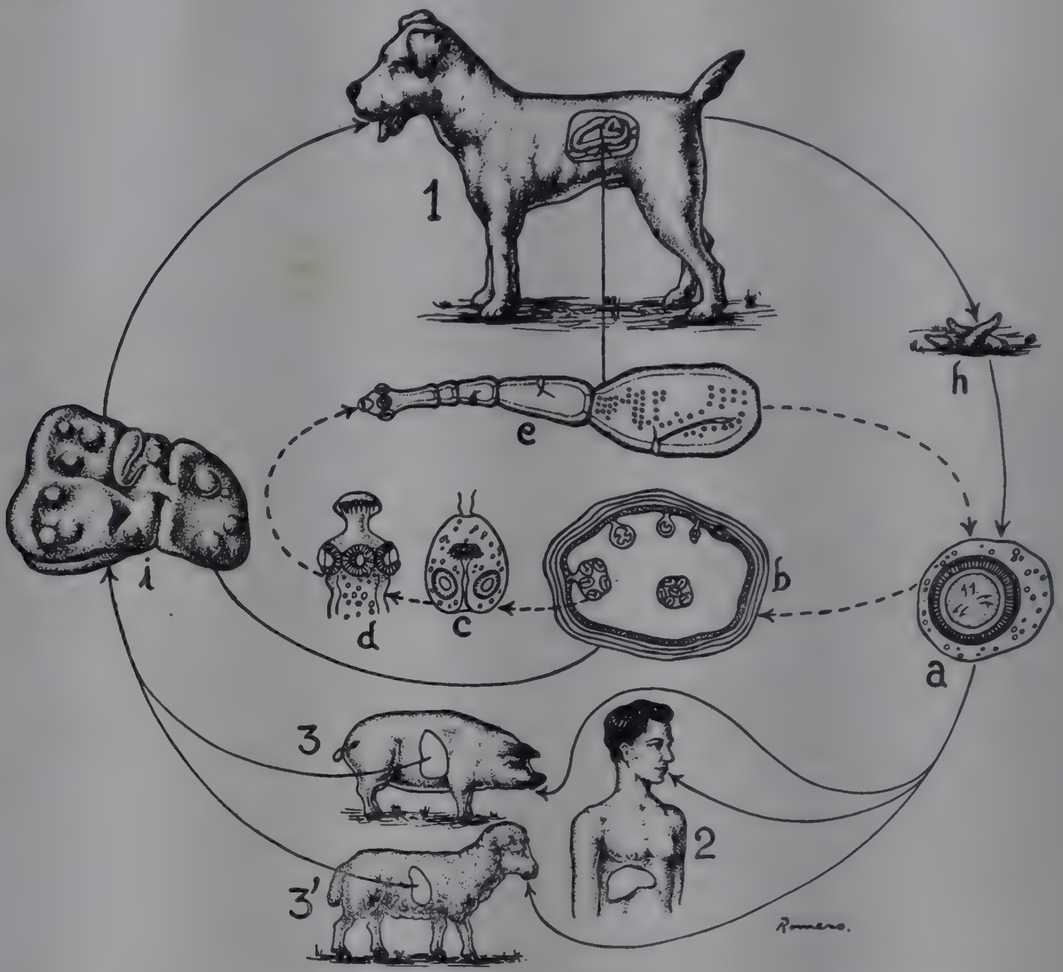


Fig. 531.—*Echinococcus granulosus.* Life cycle. The dog (*1*) the definitive host harbors the adult parasite (*e*) in its intestines. The eggs (*a*) are passed in the feces (*h*); if ingested by an intermediate host: man (*2*); swine (*3*); sheep (*3′*); or others, hydatid cysts (*b*) develop in the liver (*i*) and other viscera. When the infected viscera are fed to dogs, the scolex (*c, d*) within these hydatid cysts (*b*) develops into the adult parasite (*e*) in the intestines of this habitual definitive host, the dog (*1*), in the feces of which (*h*) eggs (*a*) are passed, beginning a new cycle. (Original drawing of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

Precipitin Test.—A mixture of 0.5 c.c. of patient's serum and 0.5 c.c. of hydatid fluid in a small test tube, placed in an incubator at 37° C. for 2 hours, gives a precipitate in positive cases. Positive reactions occur only in one-third of the cases of hydatidosis, and at times it is positive in the absence of the disease.

Complement Fixation.—This is a group reaction. Several antigens may be used: extract of *T. saginata*, of *Fasciola hepatica*, and fluid from *Cysticercus tenuicollis*. Some flatworm infestations give positive reactions with this test. The serum becomes negative within two months after removal of the hydatid cyst. About 50 per cent of cases of hydatidosis give positive results.

Intradermal Test.—Inject into the skin of the arm 0.5 c.c. of pure hydatid fluid less than six months old. Inject 0.5 c.c. of physiologic saline or weak peptone water into the other arm as a control. An accelerated positive reaction shows a wheal about 2 cm. in diameter at the point of inoculation, surrounded by a swollen area, reaching maximum intensity 15 minutes after injection and disappearing shortly afterward. A delayed positive reaction appears 12 hours after injection, and shows an erythematous area with edema and local sensation of heat.

Family **HYMENOLEPIDIDAE** Railliet and Henry 1909

Hymenolepididae are Cyclophyllideae with scolex usually provided with a rostellum bearing a single crown of hooklets, rarely unarmed. In some worms, the scolex is detached during an early stage of development and is replaced by a pseudoscolex. Suckers are usually without hooklets. Proglottids are broader than long.

Hymenolepis Nana (von Siebold 1852)

Hymenolepiasis nana is parasitism caused by *Hymenolepis nana*. *H. nana* is relatively common in certain countries, and totally absent from others of the same climate at the same latitude. It is found in Europe, Africa, Asia, and the Americas.

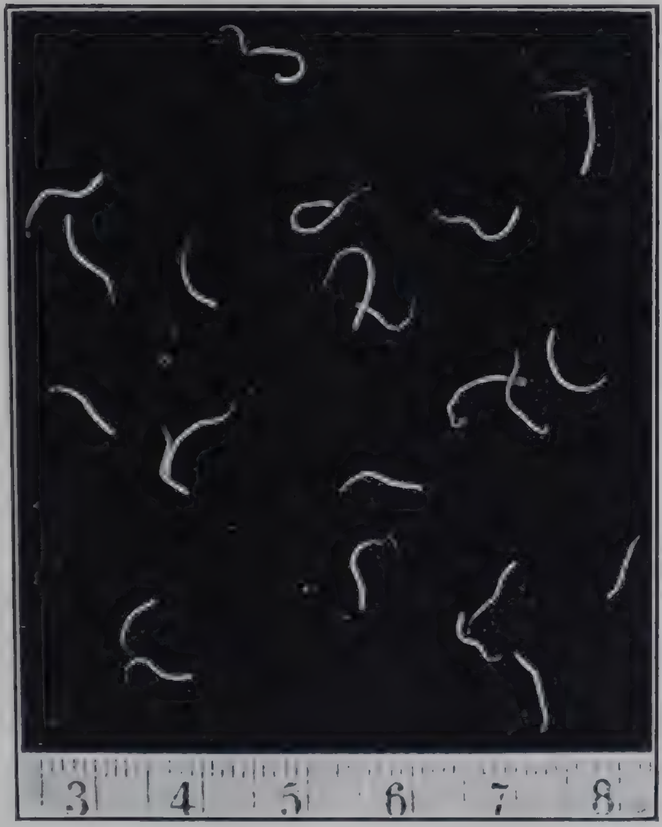


Fig. 532.

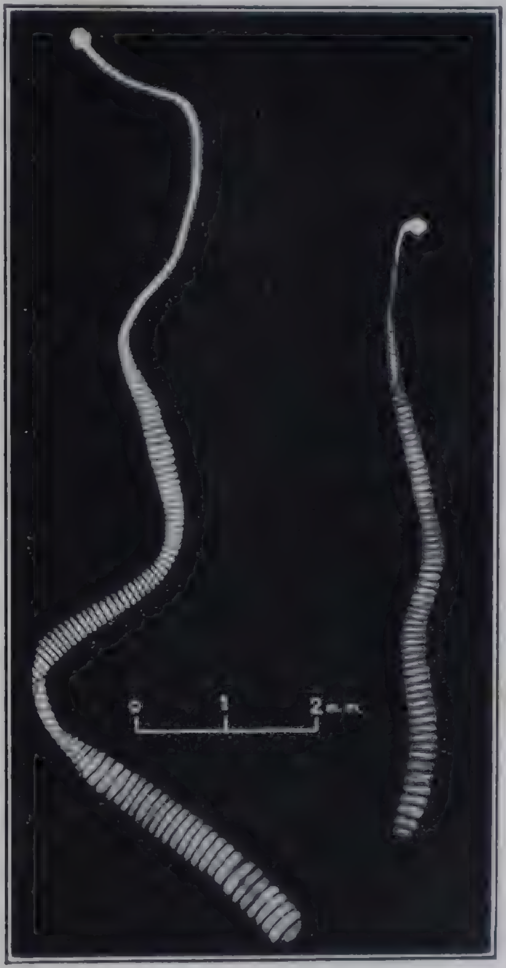


Fig. 533.

Fig. 532.—*Hymenolepis nana*. (Original photograph of Kourí, one and one-half times its natural size.) Millimeter scale. Dark background. Illumination from above. Specimens of human origin fixed in 10 per cent Formalin.

Fig. 533.—*Hymenolepis nana*. (Original figure of Kourí.) Two specimens of *Hymenolepis nana*. Dark background. Greatly enlarged. Parasites fixed in 10 per cent Formalin.

H. nana is a small worm, 25 to 40 mm. long and 0.5 to 0.7 mm. wide. The scolex has a retractable rostellum with a single crown of 20 to 30 hooklets. The neck is long.

Eggs.—The eggs are colorless and refractile, spherical or subspherical, and have two covers, in addition to the wall of the hexacanth embryo, which can be considered a vitelline embryonic membrane. The external cover is 47 microns long by 36 microns wide; the internal cover, usually oblong, is 28 microns by 23 microns, with a mamelon in each pole. From this rises a crest with 4 to 8 sinuous, refractile filaments, which occupies the space between the two covers. Within the internal cover is the onchosphere or hexacanth embryo, 26 by 20 microns. The three pairs of hooklets are lancet-shaped, 11 microns long.

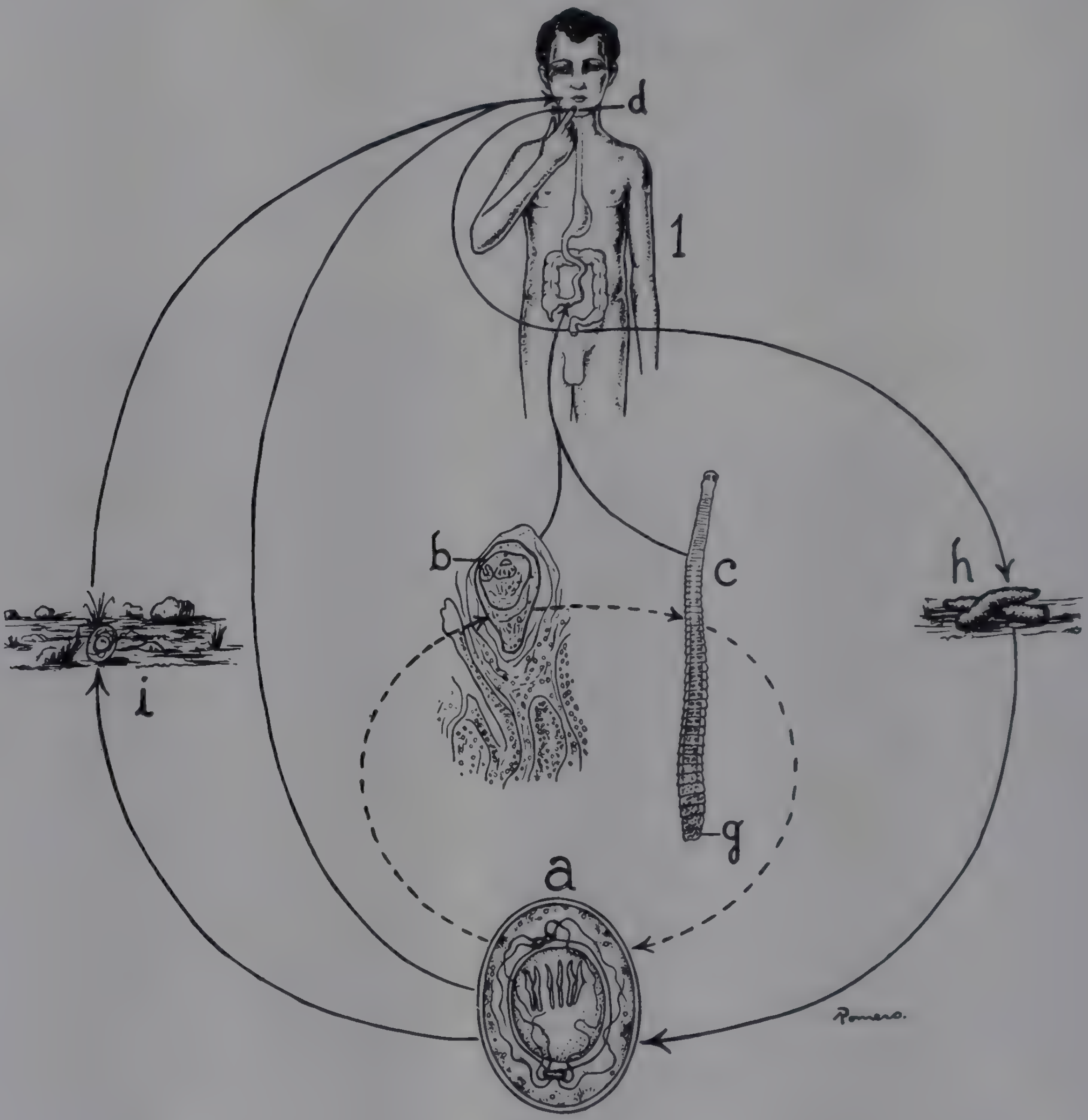


Fig. 534.—*Hymenolepis nana.* Direct life cycle: without intermediate host. Autoinfestation is present in this parasitism because the eggs are directly and immediately infective for the host. The child becomes reinfected by ingestion of eggs which have contaminated his fingers directly from the the anal or perianal regions.

The eggs (*a*) are passed in the feces (*h*); if ingested by direct contamination of the fingers (*d*) or through contamination of food (*i*), they develop into a Cysticercoid (*b*) in the intestinal villi of the same definitive host, who also acts as intermediate host by sheltering the larval form, the Cysticercoid (*b*). This Cysticercoid breaks through the villi and becomes attached to the mucosa; it grows to adult stage (*c*); the terminal proglottids of the strobila become gravid (*g*), set free eggs (*a*), which are passed in the feces (*h*) to begin a new cycle. (*b*, After Brumpt; others, original of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

The parasite is usually found in the distal portions of the ileum of man. Usually massive infestation is seen, especially in children. Two thousand parasites have been found in a single case.

Life Cycle.—*H. nana* has two life cycles. In the direct life cycle, man is both the intermediate and the definitive host. In the indirect cycle, the larvae develop in fleas (Tenebrionidae). See Figs. 534 and 535.

Diagnosis.—Diagnosis is established by microscopic examination of the feces for the presence of eggs, which are usually found in large numbers.

Hymenolepis Diminuta (Rudolphi 1819)

H. diminuta is a common tapeworm of the mouse and rat, and is also found in man. The adult is 20 to 60 cm., at times 75 cm., long, and about 3.5 mm. wide, and has a small scolex. It bears a rudimentary unarmed rostellum, with no hooklets. The proglottids are short, wide, and trapezoid.

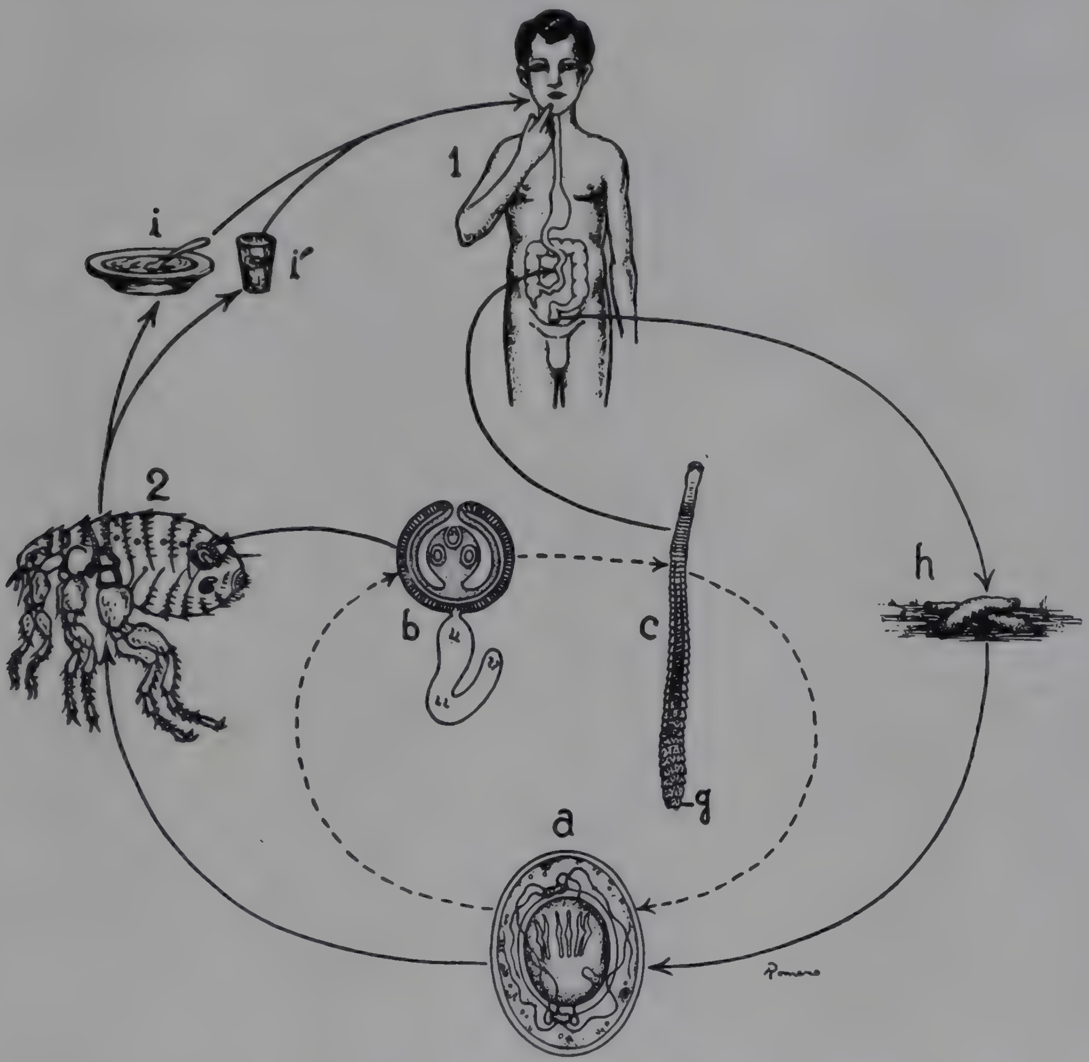


Fig. 535.—*Hymenolepis nana.* Indirect life cycle; with intermediate host. Bacigalupo has been able to infect, experimentally, rat fleas and Tenebrionidae with *Hymenolepis nana* eggs obtaining development of the Cysticercoid (*b*) in the body cavity of these insects. This leads to the thought that children may become infected through a different mechanism from that described in the direct cycle (Fig. 534); i.e., ingestion of Cysticercoid-infected fleas (*2*) which have fallen in a glass of milk (*i'*) of a plate of soup (*i*), as in the case of *Dipylidium caninum.* (Original drawing of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

Eggs.—The eggs are spherical, have two walls, and contain a hexacanth embryo. The outer wall is yellowish and radially striated. The inner wall is smooth, refractive, and colorless. The diameter is 60 to 80 microns.

Intermediate hosts are Lepidoptera (caterpillar and butterfly); Coleoptera; Orthoptera (earwig); Aphaniptera; and *Xenopsylla cheopis.*

Life Cycle.—The life cycle is indirect, requiring an intermediate host. It is shown in Fig. 536.

Diagnosis.—Diagnosis is made by microscopic examination of the feces for the presence of eggs. Direct and concentration methods should be used.

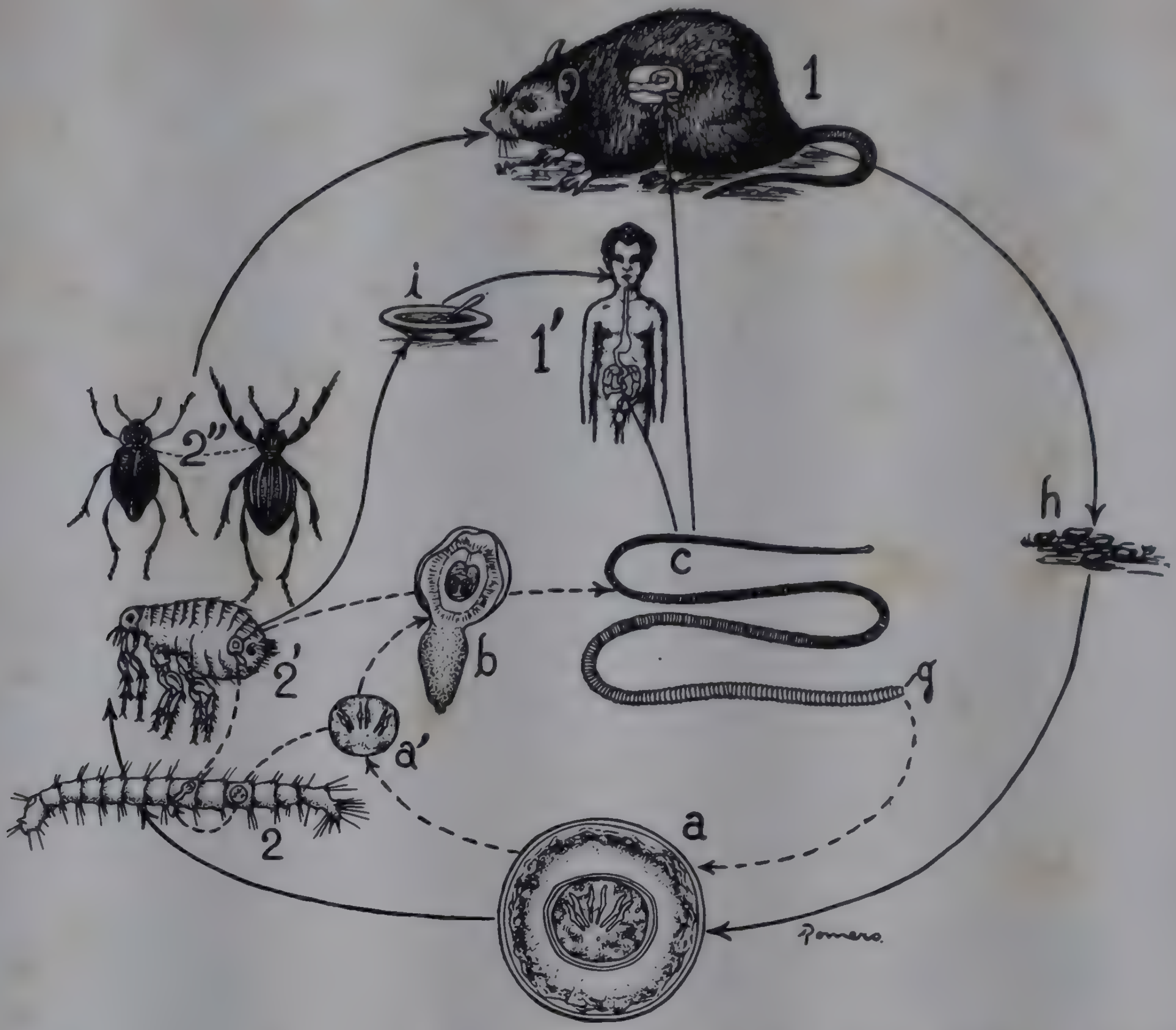


Fig. 536.—*Hymenolepis* (*H.*) *diminuta.* Life cycle. This parasite follows an indirect development. The definitive habitual (final normal) host is the rat (*1*). The child is an incidental (abnormal) host. The habitat of the adult parasite (*c*) is the small intestine of the definitive host. The eggs (*a*) are passed in the feces (*h*) of the definitive host, are freed by disintegration of gravid proglottids (*g*), then ingested by insects, as *Asopia forinalis, Akis spinosa* and *Scaurus striatus* (*2"*); rat fleas: *Xenopsylla cheopis* (*2, 2'*) and *Ceratophyllus fasciatus,* etc., in the body cavity of which they (*a*) develop into Cysticercoid (*b*). If the definitive host, rat (*1*), child (*1'*) ingests this Cysticercoid (*b*), it develops into an adult parasite (*c*) in the small intestine of the definitive host (*1, 1'*). The eggs (*a*) are passed in the feces (*h*) of the definitive host (*1, 1'*), to begin a new cycle.

The flea (*2'*) becomes infected during its larval stage (*2*). Within this larva the hexacanth embryo (*a'*) develops into Cysticercoid before the larva (*2*) becomes an adult flea (*2'*). (*2"* and *b,* After Brumpt. Others original of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.

Family **ANOPLOCEPHALIDAE** Cholodkowsky 1902

INERMICAPSIFERIASIS

Inermicapsifer Cubensis (Kourí 1938) Kourí 1940

Inermicapsifer cubensis is a cestode measuring 27 to 42 cm. in length, with 310 to 368 proglottids in the strobila. The scolex is without rostellum or hooklets, and is approximately 630 microns long and 610 microns broad. The suckers are unarmed. The neck is 3 mm. long. Proglottids of the midportion of the strobila are broader than long, about 2.3 by 1.5 mm. The free-living, gravid proglottids have variable shapes, but, when fixed in 10 per cent Formalin, they tend to become round and rather opaque.

The **life cycle** of this parasite is unknown.

The **life span** in its human host does not seem to be long, usually several months. Man is probably an accidental host. The parasite is easily expelled with the administration of castor oil or any other cathartic.

Diagnosis.—Infestation is usually discovered by the presence in the feces of small, white objects resembling rice grains. Frequently expulsion of frag-

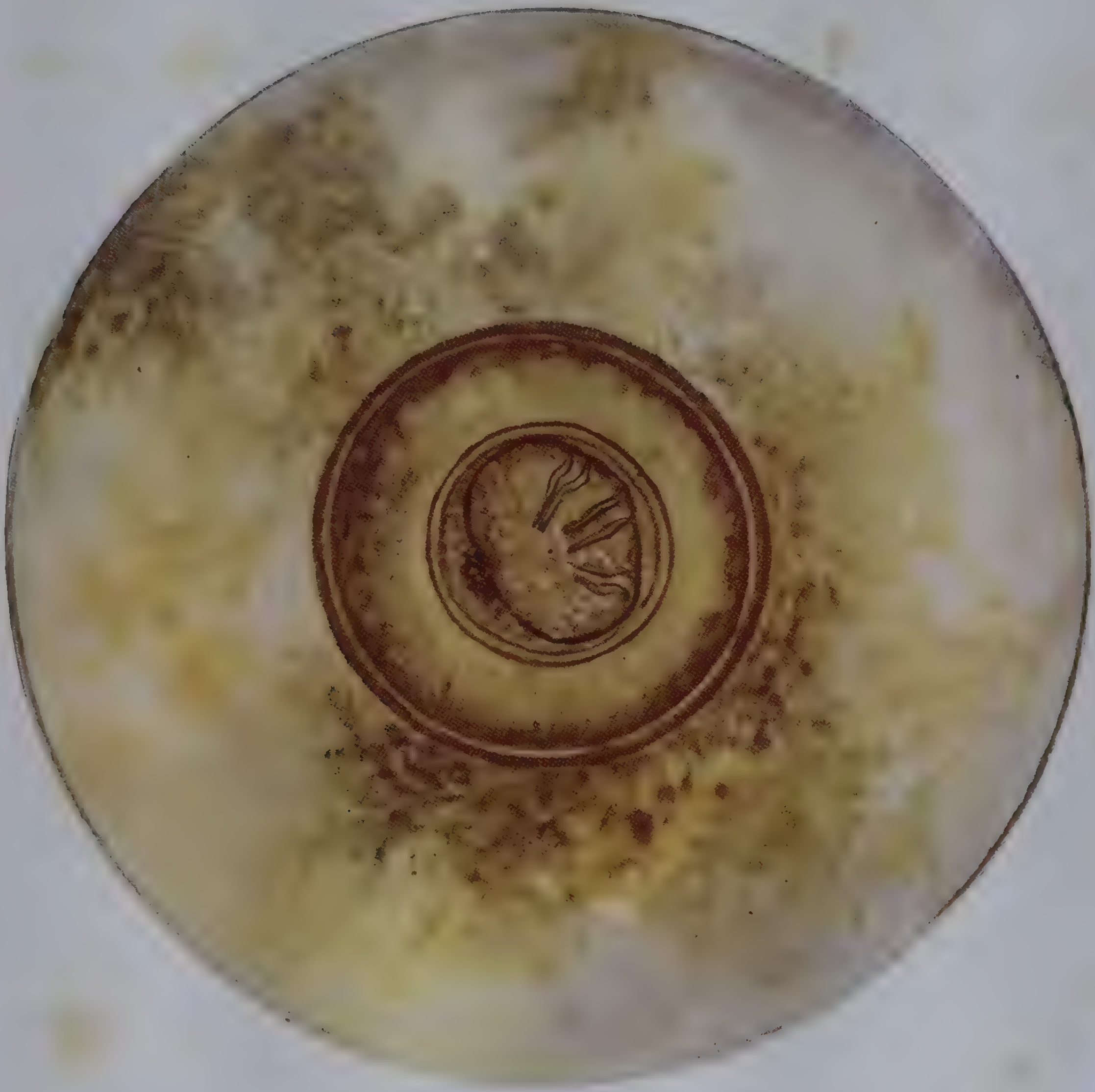


Plate LI.—*Hymenolepis (H.) diminuta* egg. (Original Figure of Kourí.) Drawn From the Microscope.

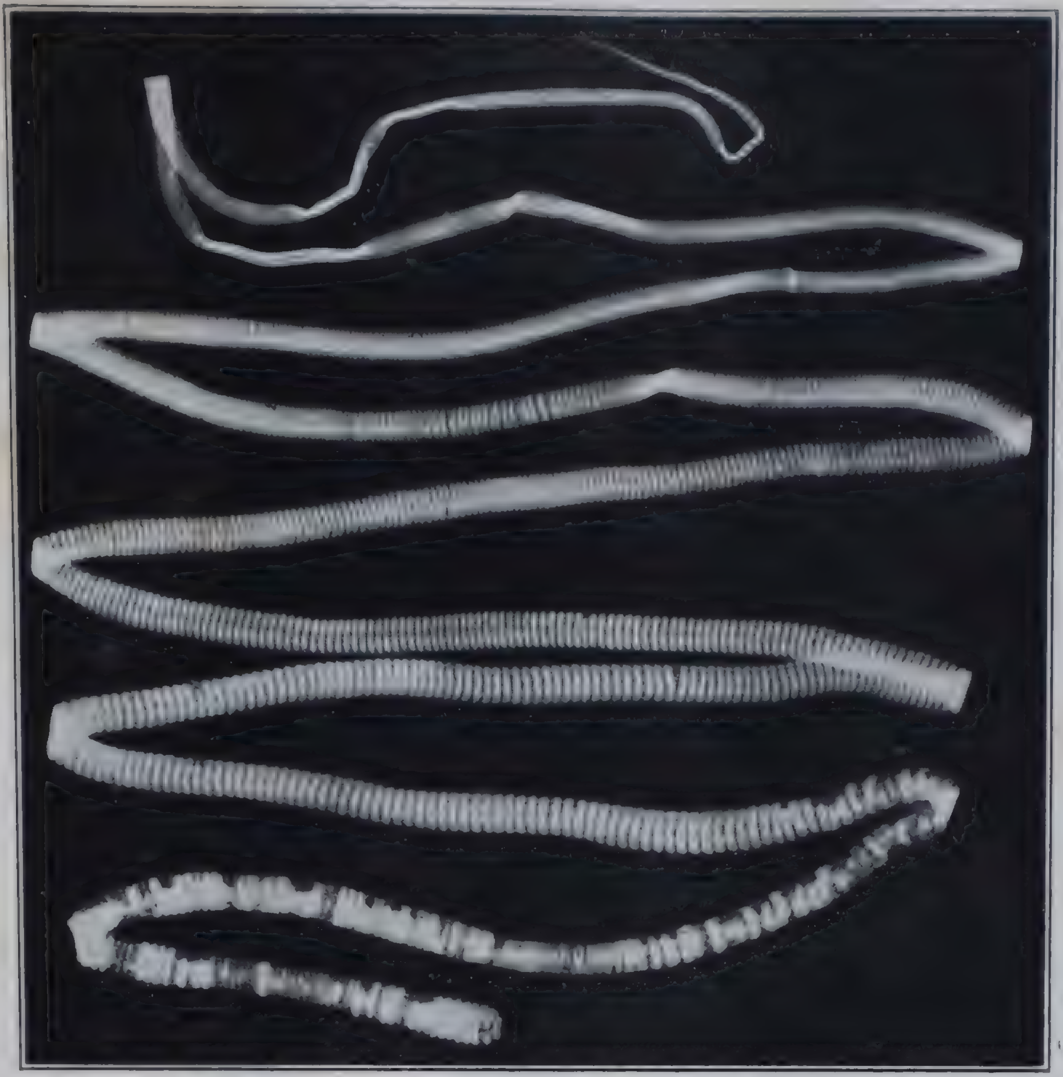


Fig. 537.—*Hymenolepis* (*H.*) *diminuta.* Specimen 75 cm. long, obtained by autopsy of a rat. The scolex is very small. All the proglottids, gravid proglottids included, are wider than long. It is difficult to see the separation between the proglottids with the naked eye. This segmentation is clearly noted if the parasite is observed with a magnifying glass, as in this photograph. (Original photograph of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

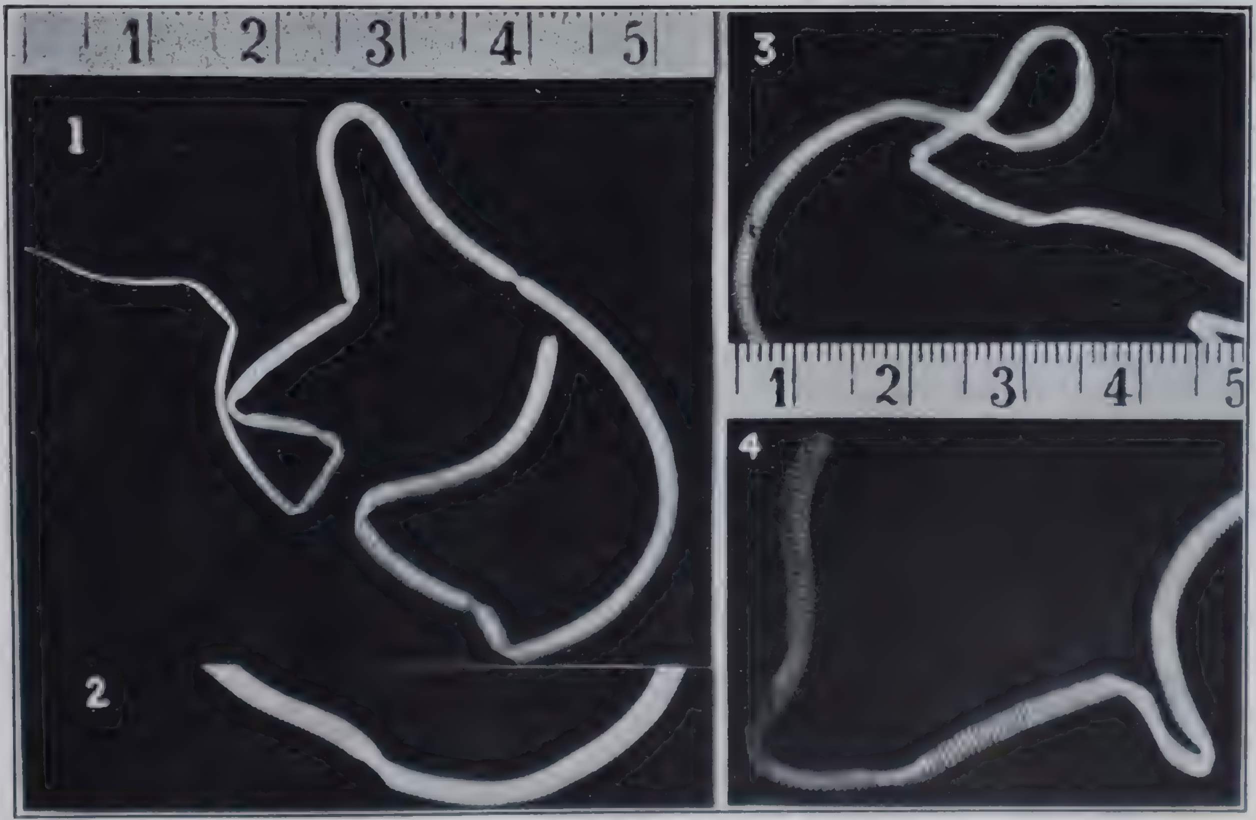


Fig. 538.—*H. diminuta.* Smaller specimen derived from rat. *1*, Complete small specimen. *2*, *3*, and *4*, Fragments of a larger specimen. Observe the finely serrated borders and the transverse striations which are very close together and resemble the segments. Dark background illumination from above. Millimeter scale. (×1.80.) (Originals of Kourí.)

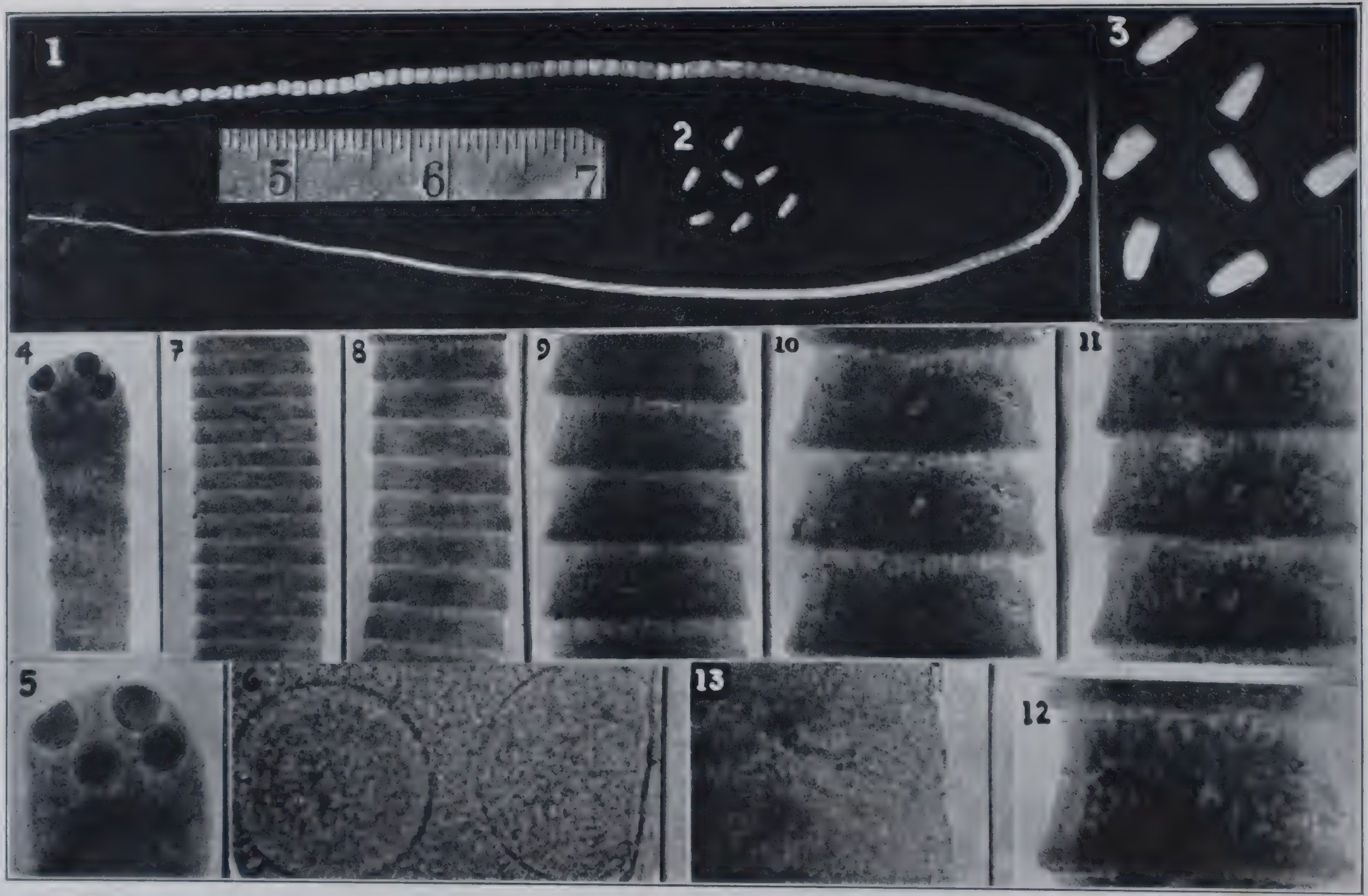


Fig. 539.—*Inermicapsifer cubensis* (Kourí 1939). *1* to *3*, Photographs, dark background, illuminated from above, specimens fixed in 10 per cent Formalin. (Originals of Kourí.)

1, Entire specimen of *I. cubensis* expelled during treatment by a 9-year-old boy. Four-fifths the natural size; scale in inches. Case treated by Kourí and Macho Doval. *2*, Mature proglottids, life size, freed from the strobila, and expelled in the feces. *3*, Same proglottid magnified. *4* to *13*, Photomicrographs of various magnifications. Specimen fixed in 10 per cent Formalin. Illuminated from below. (Originals of Kourí.) *4* and *5*, Scolex and neck. Note the two pairs of suckers. *6*, Two of the suckers, magnified. *7* to *12*, Proglottids from different sections of the strobila. *13*, Unilateral genital pores in the center of the edge of the proglottid. Small cirrus bursa.

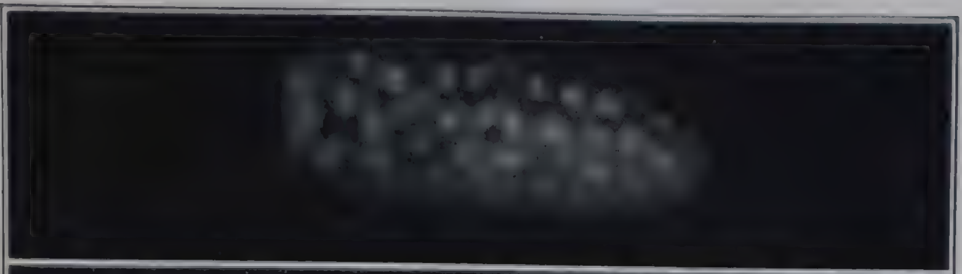


Fig. 540.

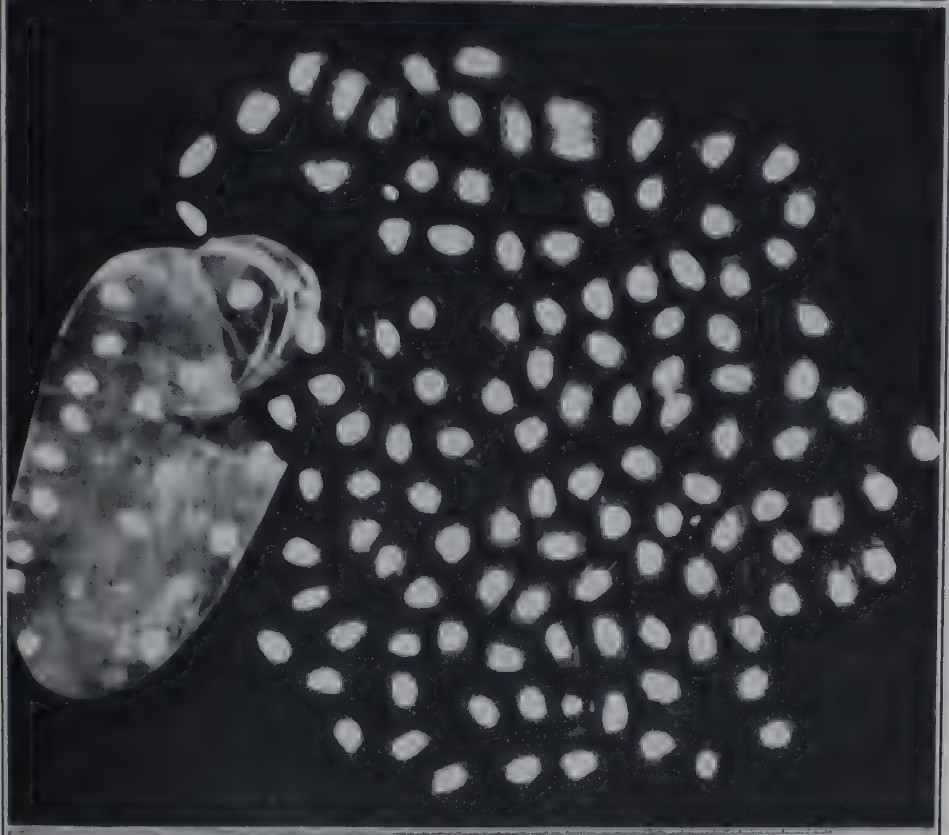


Fig. 541.

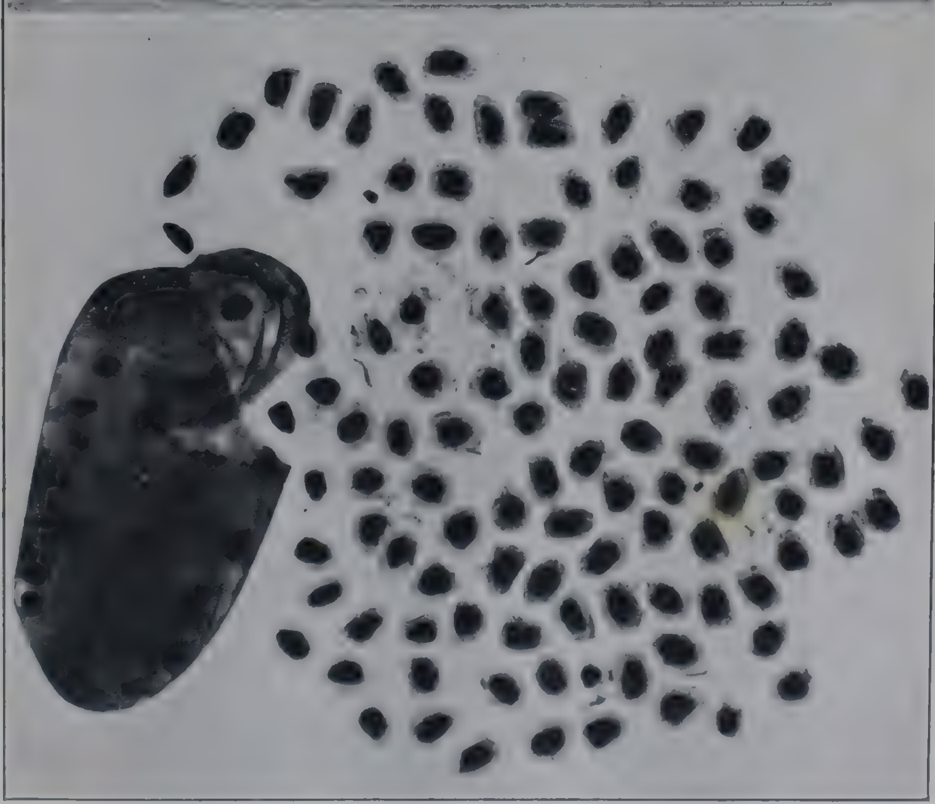


Fig. 542.

Figs. 540-542.—*Inermicapsifer cubensis,* Kourí, 1939. Gravid proglottids of *Inermicapsifer cubensis.* (Original photographs of Kourí.)

Fig. 540.—Gravid proglottid showing the egg-capsules in a cleared specimen.

Fig. 541.—The same proglottid crushed by pressure of the cover glass, expelling egg-capsules. Black background, illuminated from above.

Fig. 542.—Same as Fig. 541, but illuminated from below.

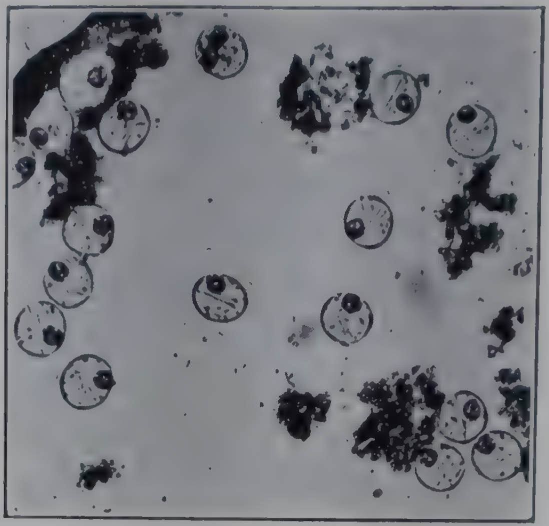


Fig. 543.—*Inermicapsifer cubensis,* Kourí, 1939. Eggs obtained by crushing egg-capsules between slide and slip cover. Medium magnification. (Material from Case No. 6 of Inermicapsiferiasis, treated by Kourí.) (Original photomicrograph of Drs. Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

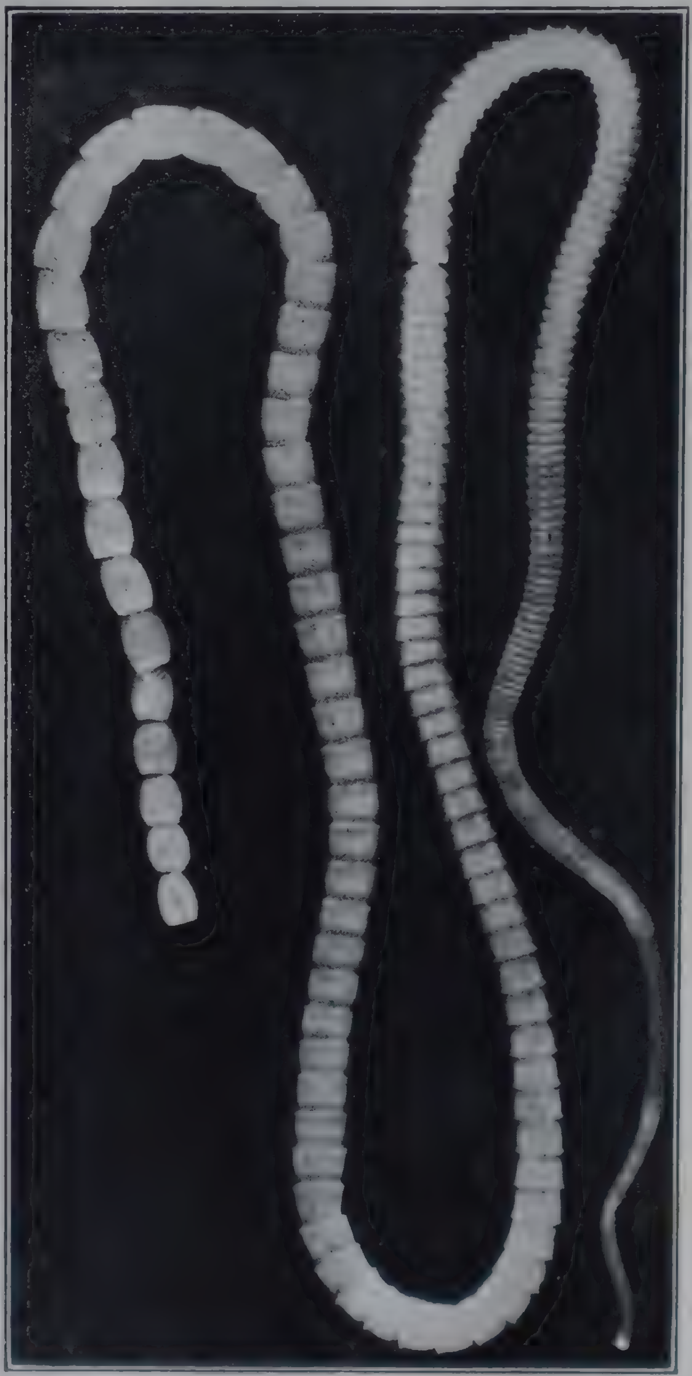


Fig. 544.—*Inermicapsifer cubensis,* Kourí, 1939. One of the complete specimens expelled, after administration of treatment, by the fifth human case of this parasitism in Cuba (Kourí). This specimen is 34 cm. long with 310 proglottids beginning the count 3.76 mm. from the scolex. (Original photograph of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

ments of strobila in the feces, either spontaneously or after the administration of cathartics, makes the diagnosis possible.

The best procedure for identification of the gravid proglottids is to examine "granules" found in macroscopic examination of the feces, by compressing these granules between slide and cover slip. If they are actually gravid proglottids, they will burst and liberate a great number of characteristic oviferous capsules. (Bacigalupo's phenomenon.) See Fig. 542.

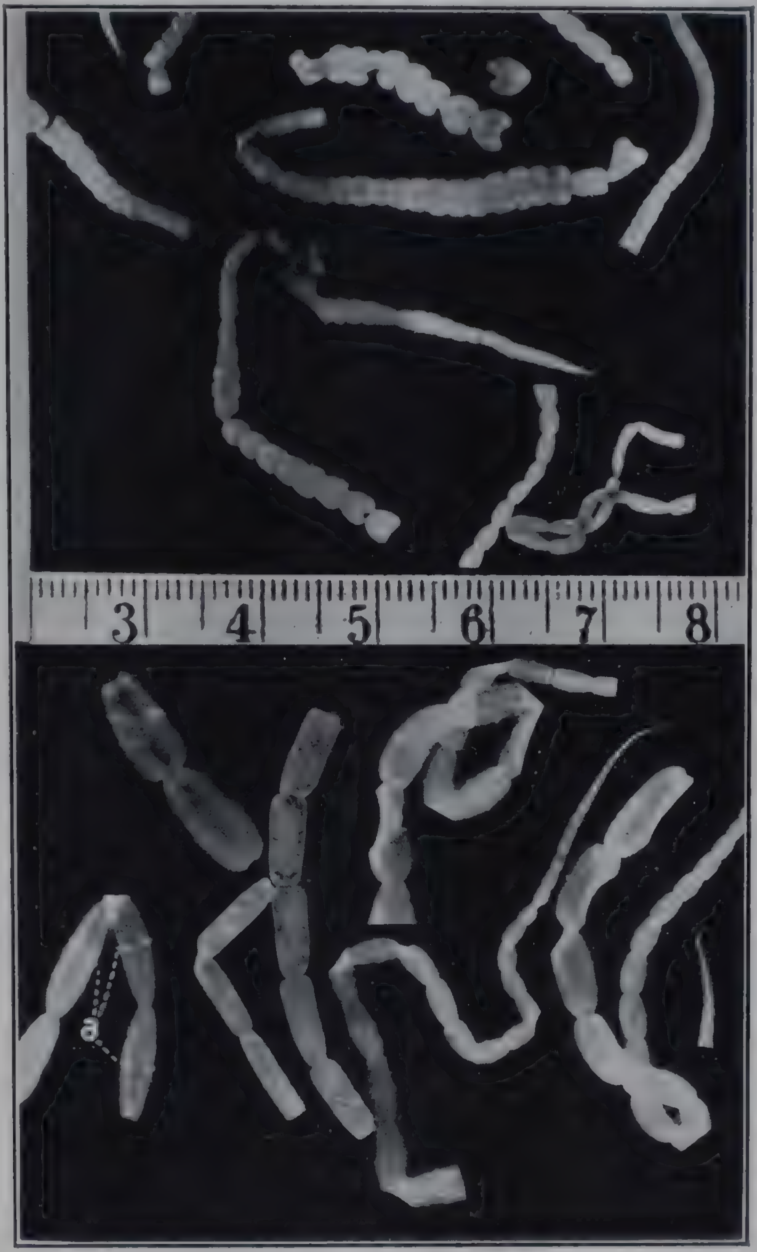


Fig. 545.—*D. caninum.* Fragments of strobila of different specimens. Note variability in shape of segments. Some are biconvex, others heart shaped. Some are long, broad, and thin; others are narrow, short, and thick. Some have almost parallel sides. In segments marked *a*, the double genital system is clearly visible. Fixed in 10 per cent Formalin; dark background, illuminated from above. Millimeter scale. (Original photographs of Kourí.)

Family **DILEPIDIDAE** Fuhrman 1907

DIPYLIDIASIS

Dipylidium Caninum (Linnaeus 1758) Railliet 1892

This tapeworm is a habitual parasite of dogs and cats, but has also been found in man. It is 50 cm. long and 2 to 3 mm. wide. The scolex is relatively small, but larger than that of *H. nana.* It has a retractive rostellum which bears 4 crowns of thornlike hooklets. The hooklets become smaller gradually from the anterior to the posterior crown which bears the smallest hooklets. There are four large, elliptical suckers. The mature and gravid proglottids are longer than wide, have convex borders, and resemble cucumber seeds.

Eggs.—The eggs are almost spherical, 52 microns long by 45 microns wide. There are two thin covers. Eggs contain a hexacanth embryo. The eggs are found grouped in numbers of 8 to 16 within the oviferous capsule, which is inclosed within the gravid proglottid.

Life Cycle.—The gravid proglottids pass outside the body and there expel oviferous capsules containing eggs. They are ingested by lice and fleas of dogs and the human flea, localize in the body cavities of the insects, and develop into larvae or *Cryptocystis*. Fleas become infested while in the larval state and the cysticercoid develops when the insect reaches the adult form. The definitive host is infested by ingesting parasitized fleas and lice.

Diagnosis.—Diagnosis usually remains unsuspected until the gravid proglottids or fragments of the parasite are accidentally discovered in the feces. The eggs and oviferous capsules do not usually appear in the feces and thus microscopic examination of the feces is insufficient to establish a diagnosis.

Order **PSEUDOPHYLLIDEA** Carus 1863

Family **DIPHYLLOBOTHRIIDAE** Lühe 1910

DIPHYLLOBOTHRIASIS

Parasitism by the adult form of *Diphyllobothrium latum* is called diphyllobothriasis or "fish tapeworm disease" or "broad tapeworm disease."

Diphyllobothrium Latum (Linnaeus 1758) Lühe 1910

D. latum is found in regions near the Great Lakes in the United States, in southern Manitoba in Canada; in Europe, the shores of the Baltic Sea and the North Sea up to Holland; the shores of Swiss and Italian lakes; and, less importantly, the delta of the Danube.

D. latum is as long as *T. saginata,* and it may be even longer. It is usually 2 to 10 meters long, but may reach a length of 15 to 20 meters. The gravid proglottids lay eggs until the uterus is empty, then contract and disintegrate. The remnants are eliminated in the feces.

Eggs.—The eggs are 70 microns by 45 microns, and may be found in great numbers by microscopic examination of the feces. They are ellipsoid, of a brownish-yellow color, and are provided with operculated coverings. The operculum is relatively large, and is found in the thicker pole of the egg.

Life Cycle.—*D. latum* inhabits the small intestines of man, dogs, cats, and other animals. The eggs are passed in the feces and eventually become deposited in water. In 11 to 15 days after reaching the water, at 15° to 25° C., ciliated embryos develop and force their way out through the opercula of the eggs, swim around in the water for 12 hours, and are then ingested by certain small crustaceans, or water fleas, called "Cyclops." In the Cyclops, the embryos develop into primary larval forms, or "procercoid" larvae, which have a caudal sphere with six embryonal hooklets called "cercomer." These larvae are ingested by fish, bore through the intestinal wall, and become encysted in various organs. Here they develop into secondary or "plerocercoid" larvae, or "sparganum." The secondary larvae in the fish pass into the intestine of the definitive host, man, dog, or cat, when the fish containing the parasite is eaten raw or poorly cooked. The scolex becomes attached to the wall of the intestines. Through proliferation of the scolex, the proglottids, and finally the complete adult parasite, are formed in 5 to 6 weeks, although after the second week eggs can be found in the stools. The gravid proglottids deposit eggs which are eliminated in the feces to start a new cycle.

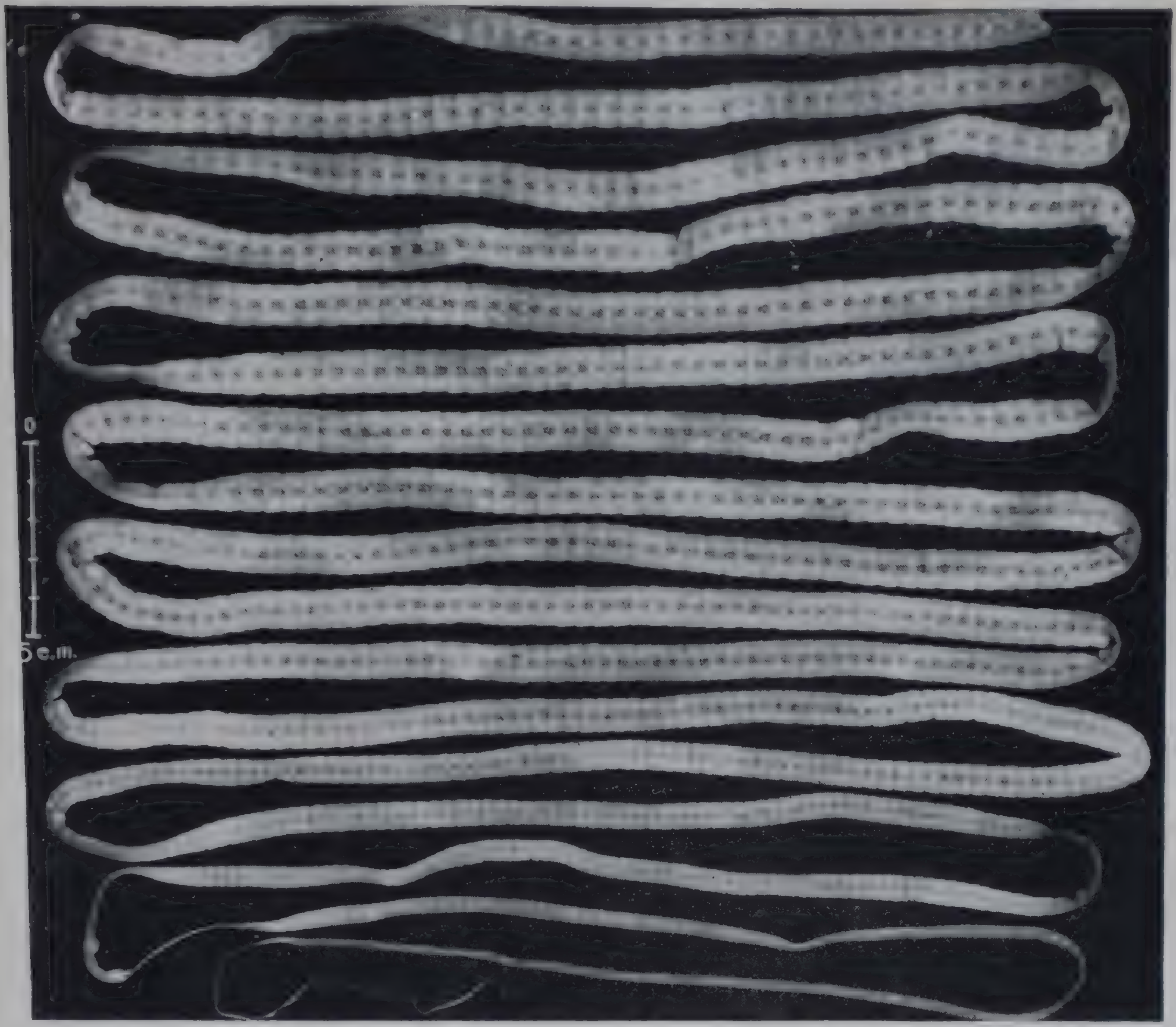


Fig. 546.—*Diphyllobothrium* (*D.*) *latum.* Complete adult specimen expelled during treatment. (Kourí, 1943, in Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

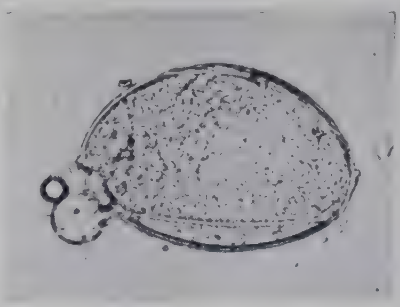


Fig. 547.—Egg of *Diphyllobothrium latum* (×400). (From the collection of R. B. H. Gradwohl.) (Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

Diagnosis.—Diagnosis is established by direct microscopic examination of the feces, where the eggs are found in great numbers. At times the patient expels long fragments of the strobila.

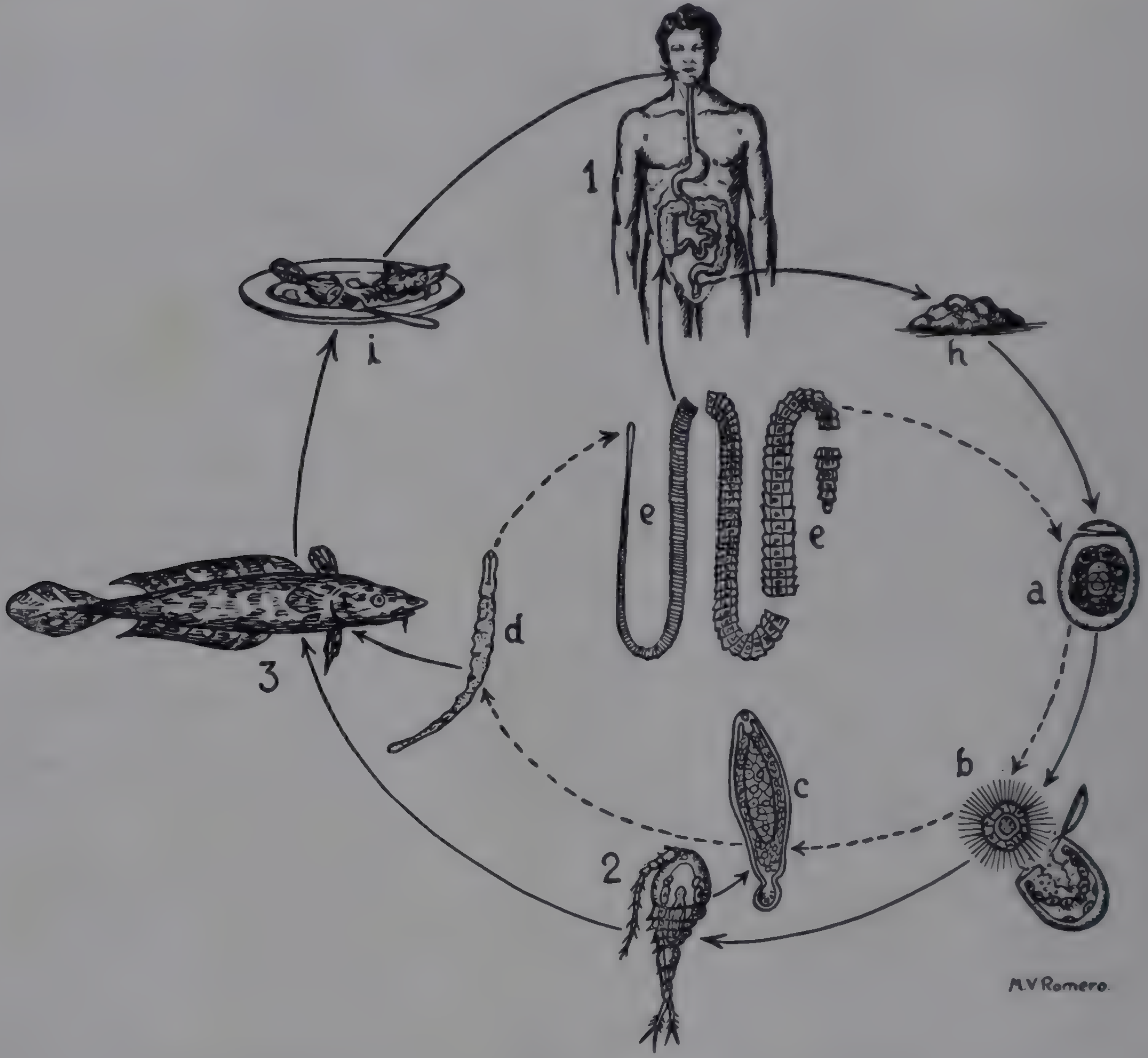


Fig. 548.—*Diphyllobothrium latum.* Life cycle. The adult parasite (*e*) lives in the small intestine of man (*1*), one of the habitual definitive hosts. It lays eggs (*a*) which are passed in the feces (*h*). If these eggs reach the water, a ciliated hexacanth embryo or coracidium (*b*) develops, hatches, and swims freely in the water until ingested by any of the first intermediate hosts, small fresh-water crustacea—copepods—commonly known as water fleas (*2*). The coracidium develops into procercoid larva (*c*) in the body cavity of these crustacea. If the procercoid larva, with the crustacea (*2*), is ingested by different species of fish, acting as second intermediate hosts, the procercoid larva (*c*) becomes a plerocercoid larva (*d*) which localizes in the viscera and muscles of the fish. When the habitual definitive hosts—man (*1*), cat, dog, seal—ingest the infected fish (*i*), raw or poorly cooked, the plerocercoid larva (*d*) becomes adult Diphyllobothrium (*e*) in the intestines of the definitive host (*1*) which passes eggs (*a*) in the feces (*h*), beginning a new cycle. (Original of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.)

THE TREMATODES OR FLUKES

Trematodes are flatworms with smooth integument, unsegmented body, incomplete digestive tract, and no anus. Usually they are leaf-shaped. All except the Schistosomae are hermaphroditic.

Class **TREMATODA** Rudolphi 1808

Family **SCHISTOSOMIDAE**

SCHISTOSOMIASIS

Schistosomiasis is a disease caused by the blood flukes. The species *Schistosoma haematobium, S. mansoni, S. japonicum,* and *S. intercalata* are the most important in human medicine.

Schistosoma Mansoni Sambon 1907

Schistosomiasis mansoni (bilharziasis) is produced by *S. mansoni*, or Manson's blood fluke. It is commonly found among the farmers of the Nile delta. It is found in many places in Africa. In the Western Hemisphere, it is found in northern Brazil, Venezuela, and Dutch Guiana, also in the West Indies. It was introduced into America through the African slave trade, and, having found an appropriate gastropod host, it became endemic.

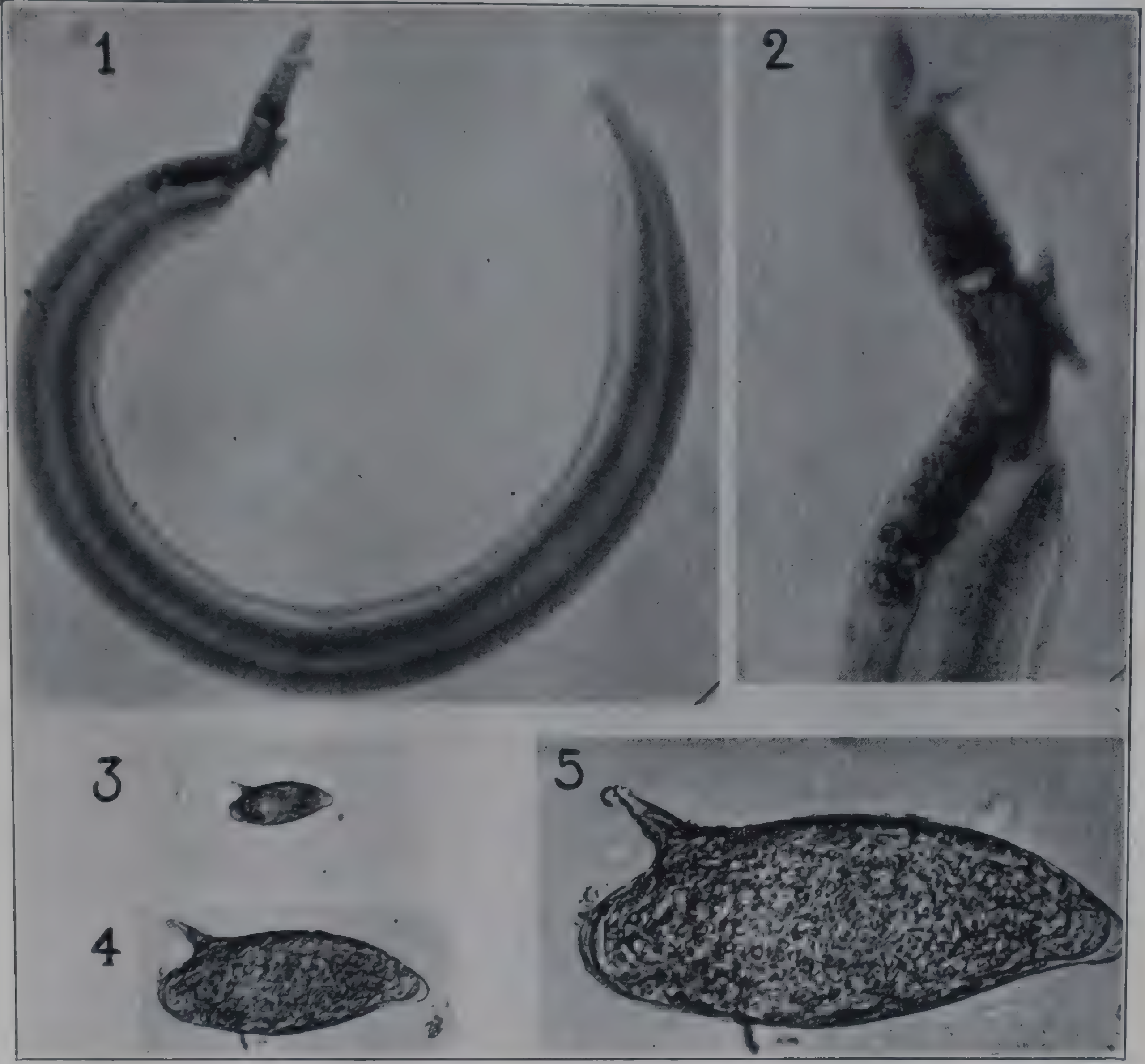


Fig. 549.—*Schistosoma mansoni.* *1*, Male parasite. *2*, Anterior part (enlarged); note the two suckers, the eight testes, and the gynecophoric canal. *3*, *4*, *5*, Photomicrographs of eggs with different magnifications. Note the characteristic lateral hook. (Original figure of Kourf and Basnuevo in Lecciones de Parasitología y Medicina Tropical.) (Photomicrograph of material sent by Dr. W. A. Hoffmann, Institute of Tropical Medicine of San Juan, Puerto Rico.)

S. mansoni causes intestinal and hepatosplenic bilharziasis. The adult parasite localizes in the venules of those branches of the portal vein which drain the colon and rectum. A small proportion of the worms localize in the blood vessels of the urinary bladder and some eggs are passed with the urine.

The female parasite is 12 to 16 mm. long and 170 microns wide. The vitelline glands occupy the posterior two-thirds of the body. The ovary lies in the anterior third. The two intestinal trunks converge, at the level of the ovary, to form a long blind pouch. The uterus is short and contains one single mature ovum at a time. The vulva is posterior to the ventral sucker which lies near the anterior (oral) sucker.

The male is 12 mm. long. It has 6 to 9 small testicular lobules. The short intestinal trunks unite into a singe blind pouch, longer than in *S. haematobium.* The body of the male is flattened and folded ventrally as in the other species of this genus. The female is held in this gynecophoric canal during fertilization.

Life Cycle.—The female migrates toward the small venules which traverse the wall of the large intestine, and deposits eggs, one at a time, into these venules. The eggs follow the capillaries, penetrate into the tissues, eventually reaching the lumen of the intestine, from which they are excreted in the feces. The eggs are carried to the water in the feces, where, at a temperature of 30° C., the embryo can usually escape from the egg within a few minutes. The larvae or miracidium has positive phototropism. It needs certain species of lung-bearing mollusks of the genus Planorbis to complete its life cycle. The miracidium penetrates, in 15 minutes, through the tentacles of the snail, and metamorphoses into a sporocyst. The sporocyst gives rise to a daughter sporocyst at the end of the fifth or sixth day. In about 10 days, the daughter

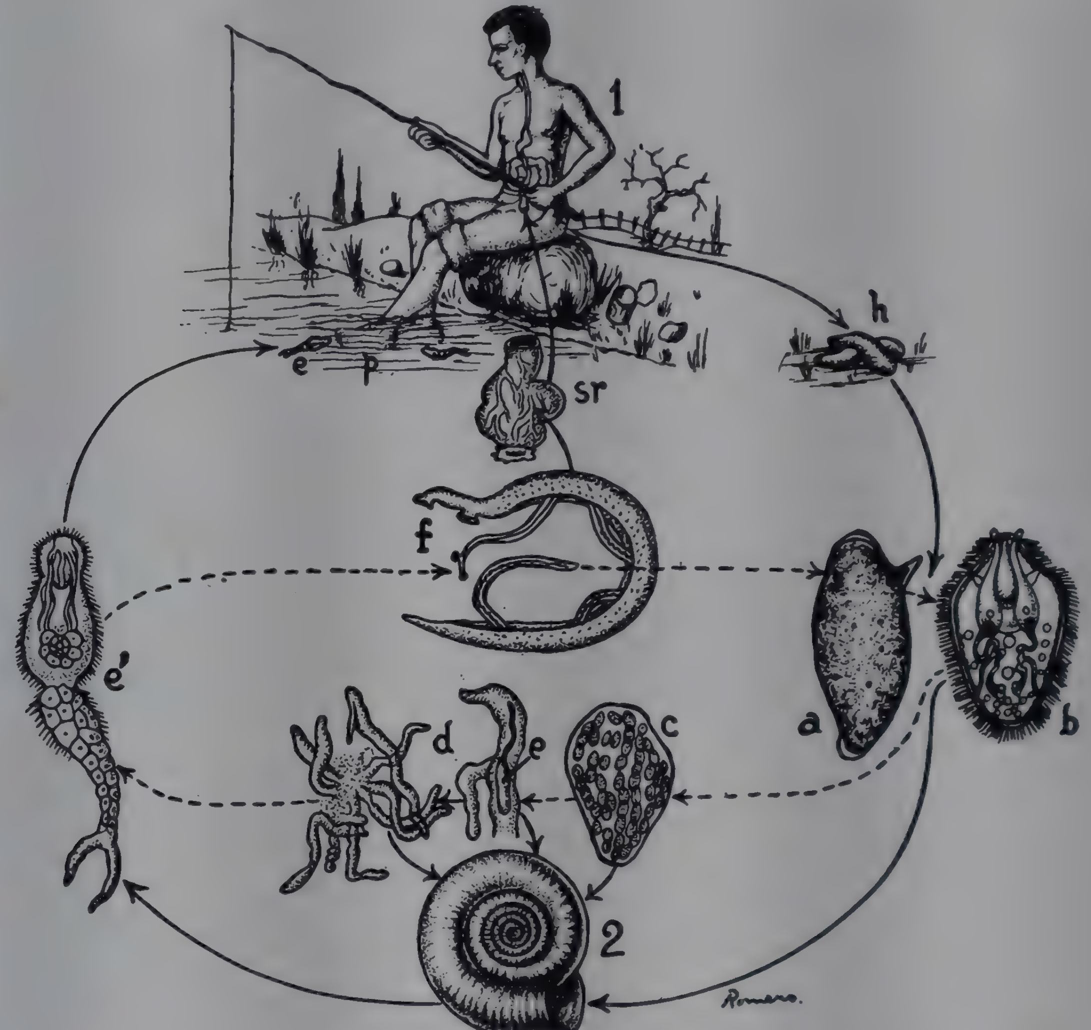


Fig. 550.—*Schistosoma mansoni.* Life cycle. The parasite lives in the veins, especially the venules of the mesenteric veins. Before laying eggs, the female migrates to the veins of the wall of the large intestine. These eggs bore through the tissues and fall in the intestinal lumen; they are passed in the feces (*h*). When the eggs (*a*) reach the water, the miracidium (*b*) hatches in a few minutes. This miracidium penetrates into different species of Planorbis (*2*) which act as intermediate hosts. The primary (*c*) and secondary (*d*) sporocysts develop within the Planorbis and the cercaria (*e*) grows within the secondary sporocyst. The cercariae (*e'*) leave the snail and swim freely in the water until they find the definitive host man (*1*), then bore actively through the skin (*p*) to reach the veins, where they develop into adult male and female (*f*). The female lays eggs (*a*) that are passed in the feces (*h*) to begin a new cycle. (*b*, After Cort, adapted by Faust; *c*, *d*, *e*, after Lutz and Leiper, in Brumpt; *e'*, after Brumpt and Neveu-Lemaire. Others, original of Kourí and Basnuevo, in Lecciones de Parasitología y Medicina Tropical.)

sporocysts give rise to cercariae. The cercariae are about 0.5 mm. long. When they come in contact with the skin of man, they shed their tails and then bore through the skin. The female lays eggs which are passed in the feces, and a new cycle begins.

The eggs are deposited by the female in the coats of the large intestine, causing "intestinal bilharziasis," or a type of splenomegaly resembling Banti's disease.

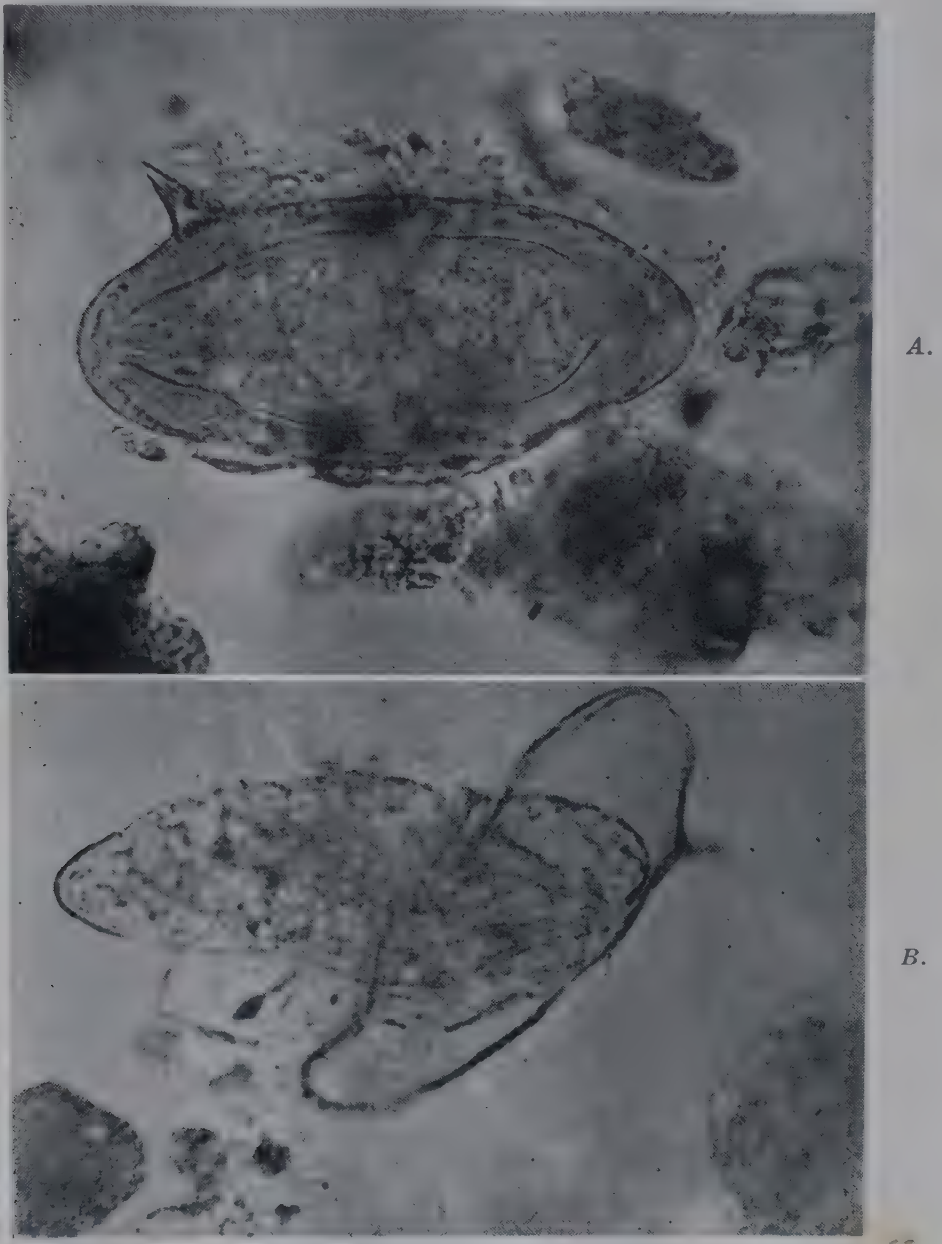


Fig. 551.—Eggs of *Schistosoma mansoni*. *A*, Egg; *B*, miracidium of *S. mansoni* emerging from the egg shell. Water centrifugal sedimentation concentration method of Baroody and Most. (Original photomicrographs of Dr. B. J. Baroody.)

Eggs.—The eggs have a prominent lateral spine. They average 142 microns in length. They usually contain living larvae.

Diagnosis.—Diagnosis is made by microscopic examination of the feces for the eggs with their characteristic lateral spine; biopsy of the lesions caused by the parasite in the rectum, rectal polyps; complement fixation reaction, using an antigen prepared from extracts of the adult parasite or of the larvae; intradermal reactions using antigen prepared from parasitized mollusks.

Rectal biopsy is useful in making a diagnosis. The technic consists in removing, with the biopsy forceps, a piece of mucosa about the size of a large grain of rice, from the right dorsoventral rectal fold, located approximately 8 to 10 cm. from the anus. The sample is then placed in 5 c.c. of a 4% solution of potassium hydroxide and incubated for 3 to 4 hours at 60° to 80° C. The tissue is dissolved in this manner and the eggs can be looked for in the sediment left after centrifugation.

Hernandez-Morales and Maldonado Technic[1]

Hernandez-Morales and Maldonado reported their results with this technic, with certain modifications, over a period of six months. Patients were asked to come to the outpatient department in the afternoon. No purgatives or enemas were recommended unless the patient was hospitalized. The rectoscope was introduced according to the usual technic. The first rectal valve was visualized, and feces, if present, were removed with a cotton swab. From the edge of the fold, a small piece of the rectal wall was pried loose with biopsy forceps, and the site inspected for any bleeding. This was controlled, if present, by pressure with a piece of cotton. The piece of tissue was pressed between two microscope slides with the help of Hoffman clamps, and examined by low power. The eggs can be easily seen and counted, and the live ones can be readily distinguished from the dead ones.

The method is based upon the fact that the *S. mansoni* lays its eggs principally in the thinner branches of the veins of the sigmoid and rectum of man. This fact was corroborated by finding, in one of the untreated cases, an adult female worm in the part of the submucosa that is usually included in the biopsy. This is one of the rare instances in which the parasite has been proved to migrate to such sites to lay its eggs. This method has proved the infection present in 100% of untreated cases of schistosomiasis against 40 and 62%, respectively, obtained through repeated examinations by the DeRivas method and the intradermal test. This test requires only a few minutes.

On several occasions, when stools had been repeatedly negative, the eggs were found by this method. It was also found by this method that the eggs could be divided into three major types: (1) The most common type is the egg which appears heavy and black under the microscope. (2) When the empty shell is found, since the yellow, chitinized remnant of the shell is all that remains of the egg, the eggs are collapsed or very transparent, and may be easily overlooked by the untrained eye. (3) The least common are the live eggs which appear just as in the sedimented stools. The miracidium is clearly outlined, with the flame cells active.

Another type of egg which cannot be placed under any of the above types is one that appears to have been recently laid, with both poles filled with granules that do not show any Brownian movement, and a clear central area. It is believed that these eggs are laid while still immature and therefore have failed to develop further.

Some eggs appear encircled in a pseudotubercle, either singly or in masses.

Schistosoma Haematobium (Bilharz 1852) Weinland 1858

The adult parasites usually live in the bladder and pelvic venous plexuses, although they may be found in the branches of the portal vein and exceptionally in other venules. The male is smaller and wider than the female. It is 10 to 15 mm. long and 0.8 to 1 mm. wide. It is covered by small tubercles and has two suckers. The body is folded from the ventral sucker to the posterior extremity to form a gynecophoric canal, within which the female lodges

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[1] Am. J. Trop. Med. 26: 811, 1946.

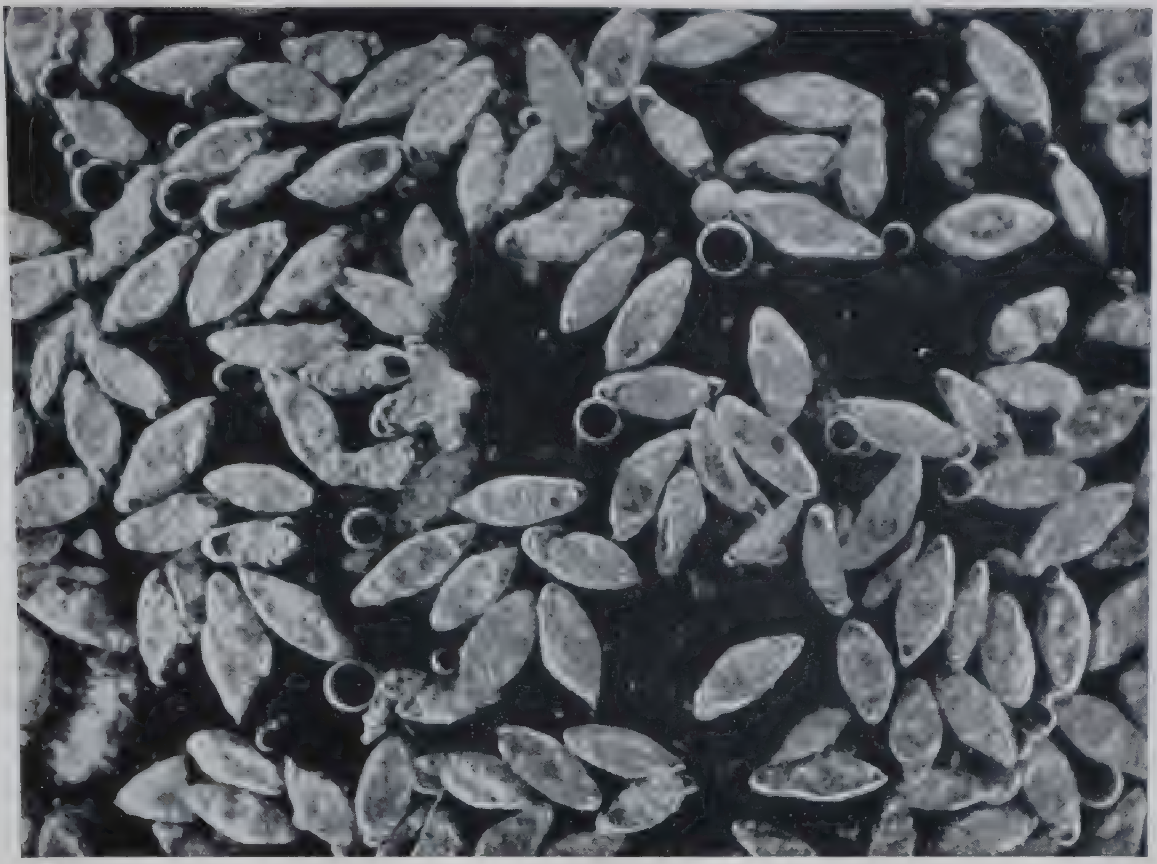

Fig. 552.—Eggs of *Schistosoma haematobium* in urinary deposit, photographed by dark-ground illumination. (Photomicrograph by D. M. Blair; published by permission of F. Goodliffe, Southern Rhodesian Public Relations Department.) (C. C. Chesterman, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

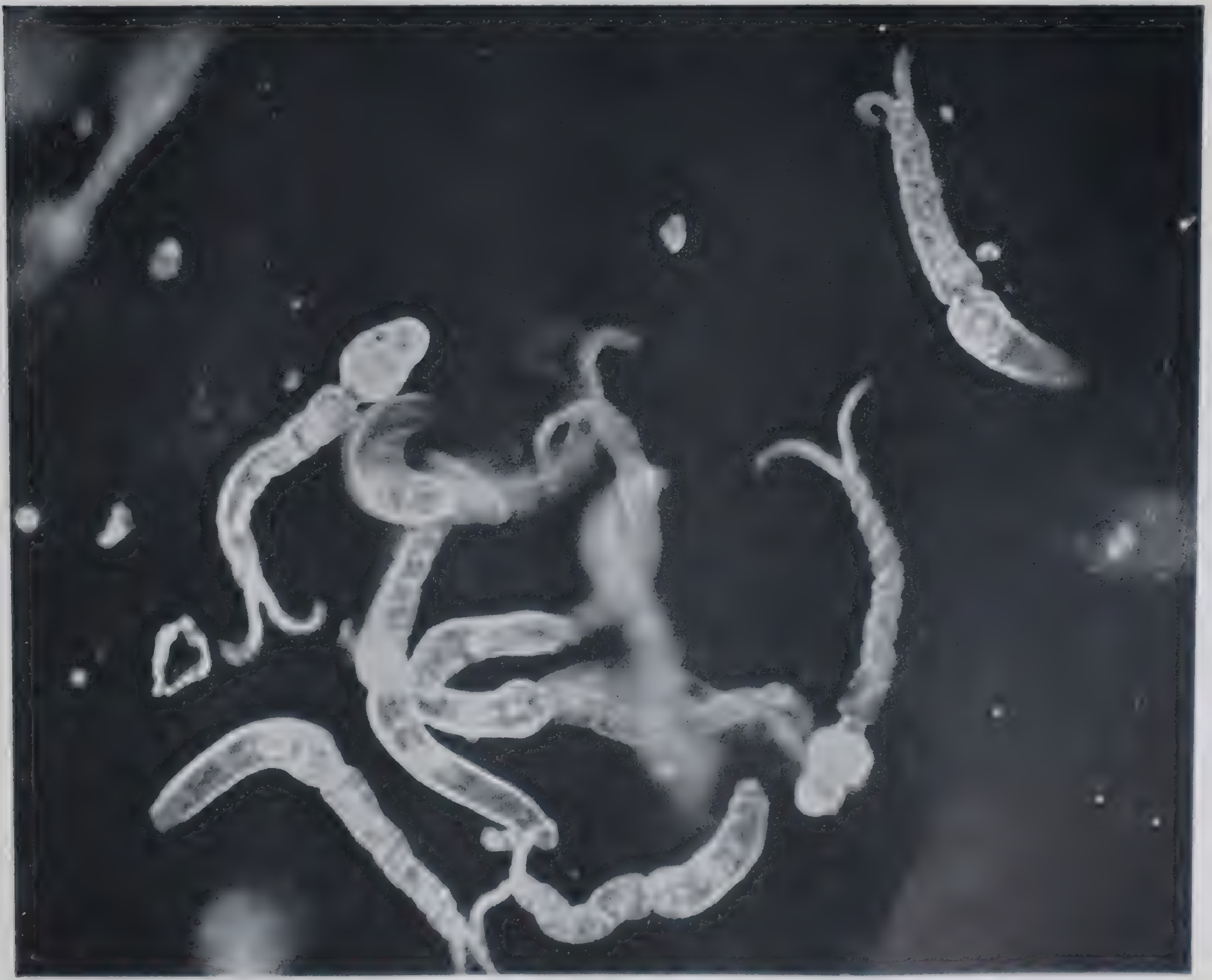

Fig. 553.—Schistosome cercariae photographed by dark-ground illumination. (Photograph by D. M. Blair; published by permission of F. Goodliffe, Southern Rhodesian Public Relations Department.) (C. C. Chesterman, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine.)

during copulation and egg laying. The intestinal ceca extend from the pharynx to the center of the body; they unite there to form a single cecum, which terminates near the caudal extremity. The digestive tract is usually dark, the color imparted by the blood which it contains. Four to five testes lie behind the ventral sucker. There is no prostatic gland or cirrus or penis.

The female is long and slender, about 20 by 0.25 mm. The suckers are small, not uniform, and not very muscular. The ovules emerge from the ovary at the posterior extremity and pass through the oviduct to the oötype, where they are fertilized. There they receive the vitelline nourishment and shell-forming substances and pass into the uterus, where they usually number 20 to 30.

Eggs.—Eggs are oval, tapering to a pointed spine at one end, blunt at the other. They average about 150 by 60 microns. They have a yellowish-brown transparent cover.

Life Cycle.—The female usually lays the eggs in the small veins of the bladder or pelvic plexus, and assumes such position that the eggs are deposited with their spines directed backward, one following the other, until the small vein is completely filled. The female then migrates to another small vein to repeat the process. The eggs are almost mature when first laid; they bore through the walls of the vein and bladder mucosa and drop into the lumen of the organ in the extravasated blood. They are passed in the urine usually at the end of micturition. The miracidia leave the eggs when the urine is diluted more than 10 times, and swim about freely. They produce in the snail, their host, two generations of sporocysts, and finally the furcocercariae. These leave the snails in great numbers. When they leave the snail, they swim actively in the water, tail forward, at times descending to the bottom of the water, and at times rising to the surface, where they remain for a short time, clinging by means of their ventral sucker.

When man bathes in these infected waters, the cercariae come in contact with the skin at the surface of the water. As the water evaporates on the skin, the cercariae bore through the skin, seeking protection for themselves. First they shed their tails. Penetration through the skin is made easy by the active movements of the anterior extremity, as well as by the secretions of certain fluids by the glands of penetration.

The metacercariae enter the small veins, are carried to the right ventricle of the heart, thence to the lungs, where they remain for several days. They then bore through the respiratory and circulatory endothelia to enter the left ventricle of the heart. From this point they enter the general circulation. They enter the mesenteric artery, pass through the mesenteric capillaries, and enter the portal circulation. In the intrahepatic portions of the portal system, the larvae begin to feed and grow. At the adolescent stage, they begin to migrate against the current of the blood, pass through the inferior mesenteric veins, and at times localize in the rectal veins. As a rule, they pass through the hemorrhoidal veins and enter the bladder and pelvic plexuses, which they reach approximately three months after they have bored through the skin.

Diagnosis.—Diagnosis is made by finding the characteristic egg of the parasite in the urine, or exceptionally in the stools.

Schistosoma Japonicum Katsurada 1904

Schistosomiasis japonica is parasitism caused by the *Schistosoma japonicum* or the Oriental blood fluke, and is also called "Katayama disease." This disease occurs in south Japan, in the basin of the Yangtze River, and around Canton, parts of Yunnan Province, and in the Philippines. The real endemic areas in Japan are the Tone River, the Kofu, the Numazu, the Fukuyama, and the Kurume areas.

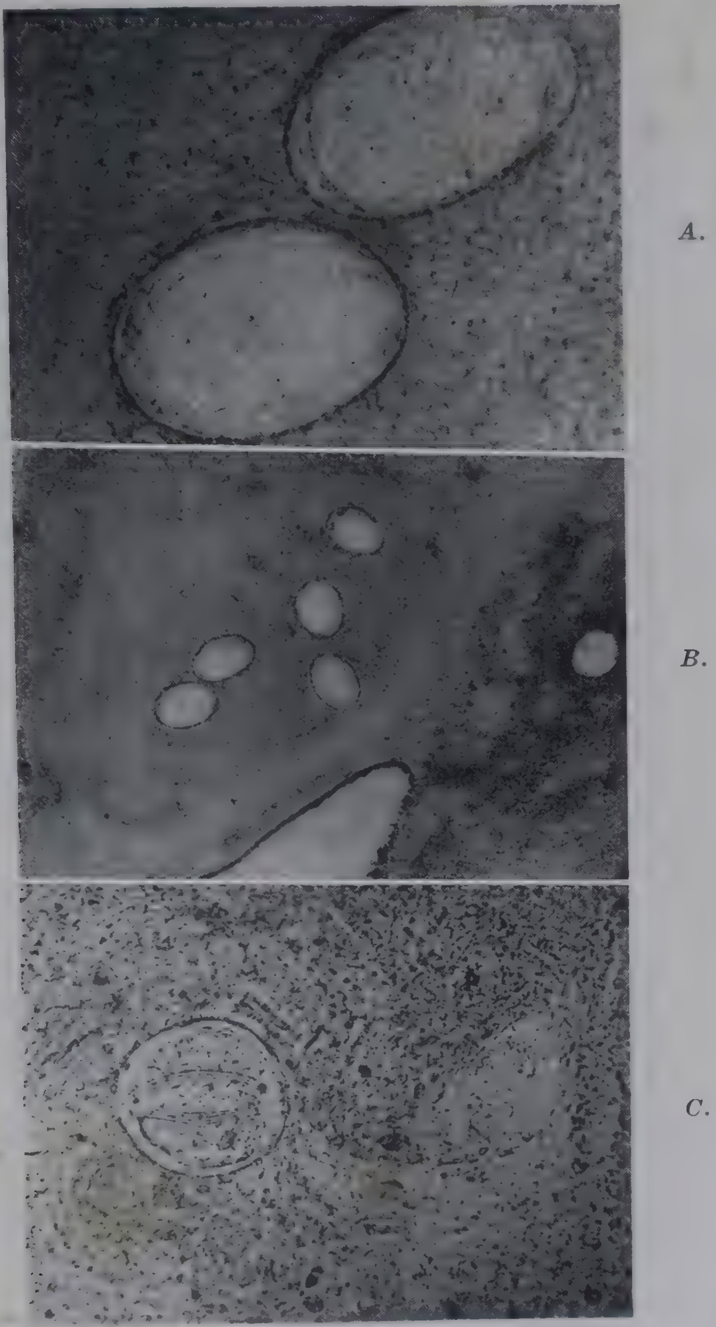

Fig. 554.—*Schistosoma japonicum* eggs. *A*, In bloody mucus of stool specimen; *B*, "nest" of eggs in bloody mucus; *C*, egg shell of *S. japonicum* showing typical break in the shell after emergence of miracidium—miracidium can be seen distinctly to the right of the egg shell. Water centrifugal sedimentation concentration method of Baroody and Most. (Original photomicrographs of B. J. Baroody.)

The adult *S. japonicum* resembles the *S. mansoni* and the *S. haematobium*, but it lacks the tubercular integument. The male is 12 to 20 mm. long and 0.5 to 0.55 mm. wide. It has an integument covered with small spines, more prominent near the suckers and the gynecophoric canal. There are 7 testes.

The female is about 26 mm. long and 0.3 mm. wide. It contains an integument covered with spines. The ovary lies behind the center of the body.

Vitellaria are limited to the lateral fields of the posterior quarter. The oötype lies immediately in front of the center of the body. The uterus is a long, straight tube, which contains up to 50 eggs at one time.

Eggs.—Mature eggs are usually but not always yellowish, discoid oval, and larger than the usual helminth eggs, varying from a minimum of 55 to 60 microns in length in the young eggs to a maximum of 85 to 90 microns in mature eggs. The shell may be covered with varying amounts of detritus, but is never completely obscured. A spine is always demonstrable in newly laid or immature eggs, but it is usually difficult to demonstrate in mature eggs.

The characteristic feature of the egg is the miracidium contained within it. It is well to call an object a schistosome egg only when both the outline of the shell and the embyo can be seen: (1) muscular twisting and jerking of the whole or parts of the miracidium; (2) ciliary action; and (3) the beating of the flame cells.[1]

The viable egg is biconvex, like the shell of a rather flat clam, and measures 58 by 44 microns. It has a noticeable wartlike thickening near one end at the site where the abbreviated spine is sometimes seen. Mature eggs show distinctive visible cilia, which are frequently in motion. Faust calls attention to immature eggs with many types and stages of degeneration, from early fertile but unsegmented stages to dead eggs which have partially or almost completely developed.

Life Cycle.—The *S. japonicum* characteristically lays its eggs in the small veins of the submucosa or even in the small veins of the mucosa of the adjacent organs. They are deposited in radicles of the superior mesenteric vein. These eggs pass from the submucosa to the mucosa, thence to the intestinal lumen, where they are expelled in the feces with some extravasated blood, and a life cycle similar to that of the *S. mansoni* and *S. haematobium* begins.

The amphibian snails which serve as intermediate hosts are *Katayama nosophora* in Japan and along the coast of China; *K. formosana* in Formosa; *Oncomelania hupensis* in the valley of the Yangtze River in China; *O. hydrobiopsis* and the *Blanfordia quadrasi* in Samar and Leyte, the Philippine Islands.

Diagnosis.—Diagnosis is made by finding the characteristic eggs of the parasite in the stools of parasitized individuals. Methods of finding these by concentration are given on pages 1805 to 1812. Blood eosinophilia, as well as the complement fixation test and clinical history of the patient, will serve in the differential diagnosis.

Differential Diagnosis.—

Needle Biopsy of the Liver.—In the chronic stage, the liver is enlarged, and remains tender, with an eventual fibrosis, and a portal cirrhosis develops. Some authors, such as Symmers, believe that the cirrhosis is secondary to the dietary conditions under which these patients live, and that the parasitic infestation is incidental. No adequate description of needle biopsy of the liver in schistosomiasis japonica has previously been found in the literature in reference to hepatic lesions. Stonehill, Cleve, and Webb[2] make an interesting observation on the diagnosis of schistosomiasis japonica by needle biopsy of the liver in the case where there is a chronic liver cirrhosis following this disease. They used a Vim-Silverman biopsy needle, and advocate this as a safe method of obtaining the specimen without exposing the patient to a laparotomy, if the usual diagnostic technics fail to reveal the organism. In their case, multiple rectal biopsies and concentration tests failed to reveal any eggs. This is in line with many reports where stool examinations fail to demonstrate the eggs. Even with rectal biopsies, failures have been reported as high as 11 per cent. With a history of possibility of exposure, needle biopsy of the liver should be considered as a means of establishing the diagnosis. A needle biopsy of the liver in the case cited by Stonehill and associates revealed the presence of an egg of *S. japonicum*.

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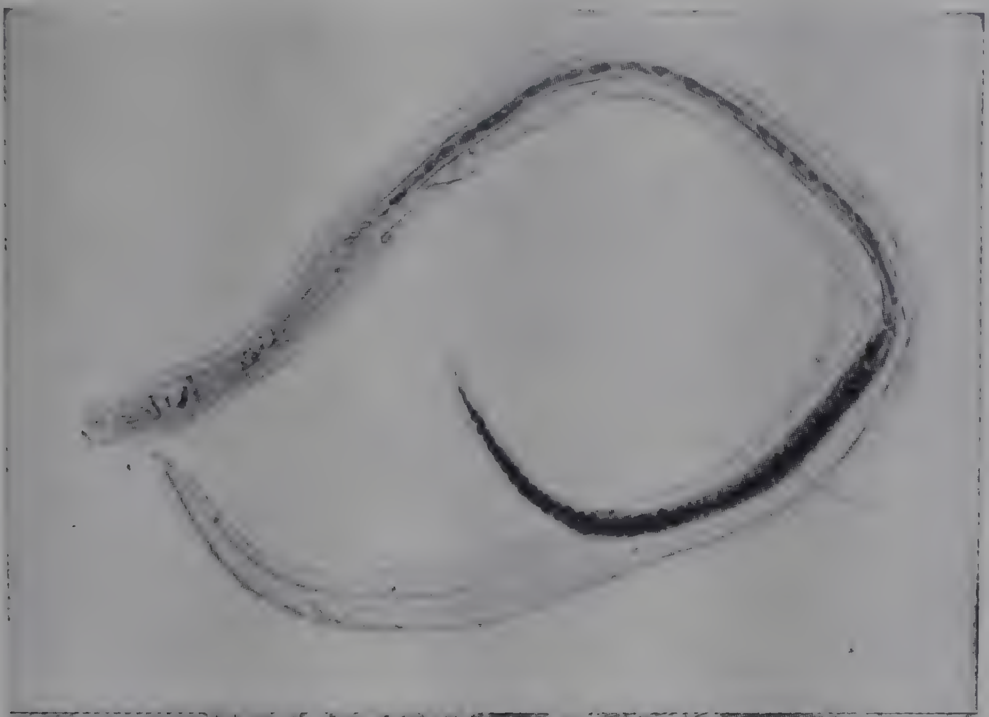
[1] Bang, in Gradwohl, Benitez Soto, and Felsenfeld: Clinical Tropical Medicine. The C. V. Mosby Co., 1951.

[2] J.A.M.A. 155: 567, 1954.

Fig. 555.



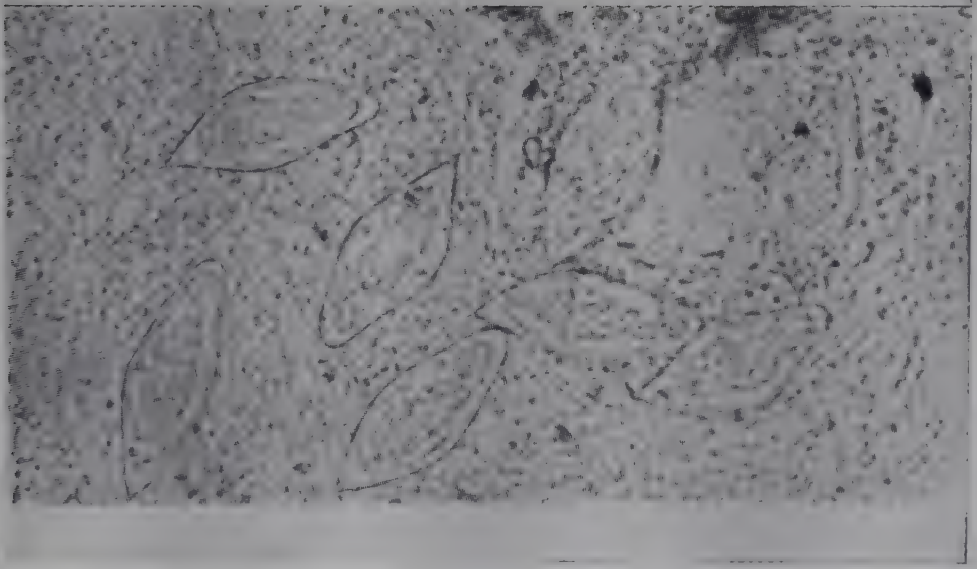


Fig. 556.

Fig. 555.—Male and female of *Schistosoma intercalatum* in copula. Fixed by lacto-phenol. (×14.) (Experimental mouse.) Note cecum in posterior one-fifth only. (Courtesy Fisher, A. C.: 1934. Tr. Roy. Soc. Trop. Med. & Hyg. 28: 277-306; by permission of the author.)

Fig. 556.—Eggs of *Schistosoma intercalatum* in feces of man. (×135.) (Courtesy Fisher, A. C.: 1934. Tr. Roy. Soc. Trop. Med. & Hyg. 28: 277-306; by permission of the author.)

Schistosoma Intercalatum Fisher 1934 (?)

The adult worms are indistinguishable from *S. haematobium.* The male measures 11 to 14 mm. long by 0.3 to 0.4 mm. wide when not unfolded. There are 3 to 5 testes, usually 4. The mature female is 10 to 14 mm. long, with a maximum diameter of 0.15 to 0.18 mm.

Eggs.—Eggs are intermediate in shape and size between those of *S. haematobium* and *S. bovis.*

Schistosomiasis intercalata is a chronic disease caused by infestation of the inferior mesenteric veins by the adult worm; the eggs produce mild dysenteric and possibly other symptoms, but never invade the genitourinary organs. Sigmoidoscopy reveals characteristic lesions between the anal canal and the pelvirectal junction.

Diagnosis is made by finding characteristic eggs in the intestinal mucus, which can be scraped from the surface of a fecal mass or obtained by means of a rectal swab or curettage through a proctoscope.

DERMATITIS PRODUCED BY NONHUMAN SCHISTOSOMES

Cercariae of the Schistosomes which infect man, when boring through the skin, frequently give rise to a more or less marked itching. Cort in 1928 described a dermatitis produced by cercariae of Schistosoma which in the adult stage are incapable of infecting man. It is common in the north central states, as well as in other parts of the United States, in southern Canada, and in Europe. At least eight species have been reported as capable of producing this dermatitis or "swimmer's itch."

C. physella and *C. stagnicolae* have been reported in Michigan. The most important European form is the cercaria of *Trichobilharzia ocelallata* found in Germany, France, and Wales.

Cercariae penetrate the skin only when the water begins to evaporate on the swimmers.

DISEASES CAUSED BY LUNG FLUKES
PARAGONIMIASIS or ORIENTAL LUNG FLUKE

Paragonimiasis is parasitism caused by the *Paragonimus westermani.* It is also called "Oriental lung fluke." The disease originates in the Far East, where it is found especially in China, Korea, the Philippine Islands, Japan, Indo-China, and Formosa. Occasional cases have been reported in Peru, Venezuela, and the United States.

Family **TROGLOTREMATIDAE** Odhner 1914
Paragonimus Westermani (Kerbert 1878) Braun 1899

Paragonimus westermani is the causative agent of "pulmonary distomiasis," or "parasitic hemoptysis" in man. The worm is 8 to 16 mm. long, 4 to 8 mm. wide, and 3 to 4 mm. thick. It is reddish brown, and when kept in alcohol is the shape and size of a coffee bean. The oral sucker is subterminal; the ventral sucker a short distance in front of the center of the body. The suckers are almost the same size (1 to 1.4 mm.). The two blind intestinal trunks are sinuous and unbranched. The genital pore is near the posterior border of the ventral sucker. The parasite is covered with broad spines, varying in shape; those between the two suckers resemble a cluster of scales. The excretory vesicle is very large and extends to the bifurcation of the intestinal ceca.

Eggs.—The eggs are operculated, ovoid, reddish-brown, 85 to 100 microns long, and 50 to 60 microns wide. They are abundant in the sputum and less frequently found in the feces and other specimens.

The **life cycle** is found in Fig. 557.

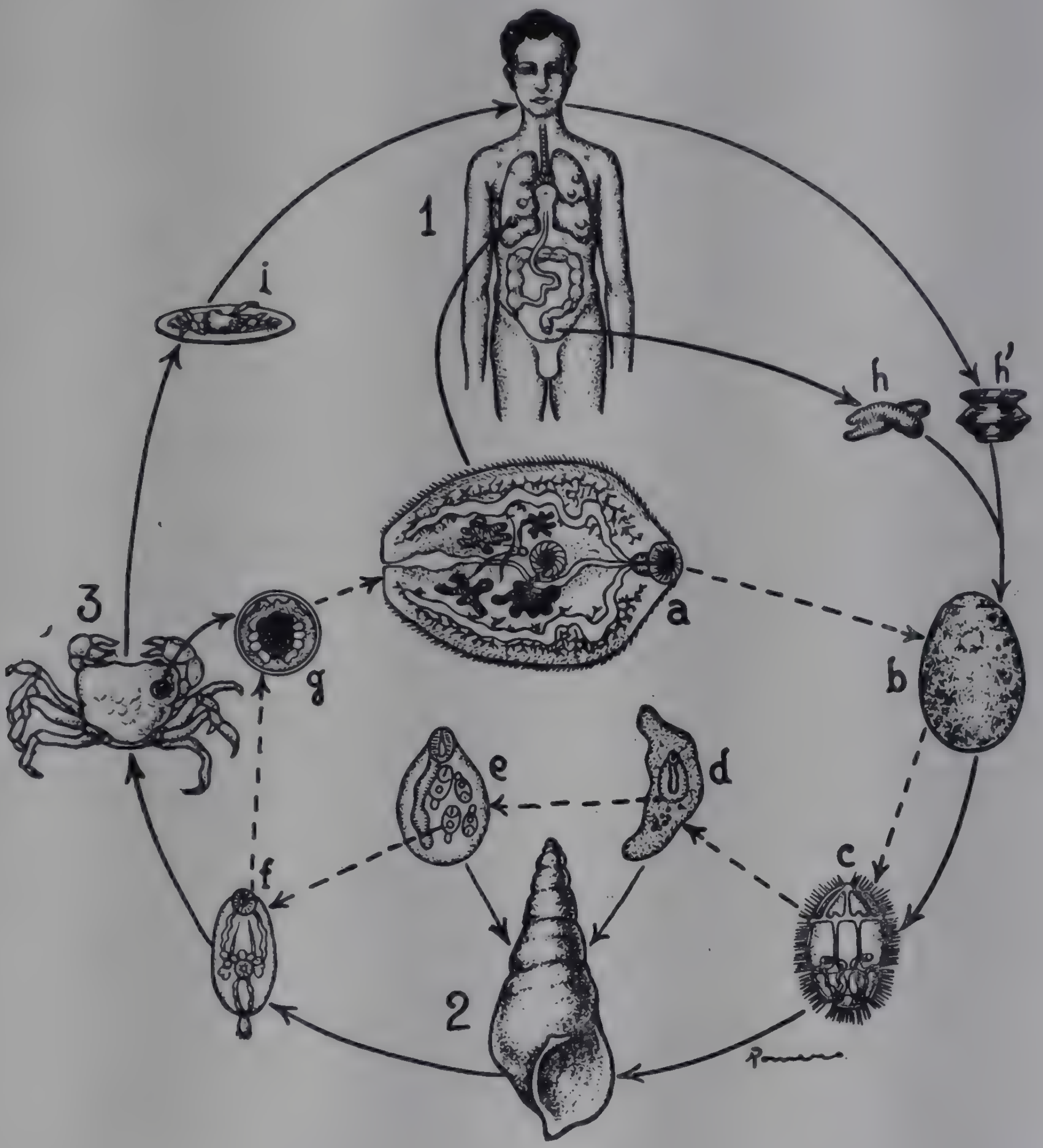


Fig. 557.—*Paragonimus westermani.* Life cycle. The habitat of the adult parasite is the lungs of the definitive hosts, of which man is one; they are found in small cystic cavities, usually in pairs. The egg (*b*) is coughed up in the sputum (*h'*) or passed in the feces (*h*), and a miracidium (*c*) is hatched in from two weeks to two months, depending on the temperature. It (*c*) bores into snails of the genus Melania, especially *Melania libertina* (*2*), one of the first intermediate hosts, develops into sporocyst (*d*) under the epidermis of the mollusk; usually only one redia grows. Daughter rediae (*e*) develop from this redia and become attached to the hepatopancreas of the mollusk, undergo parthenogenesis, and form the cercariae (*f*). These leave the snail and become attached to the thoracic muscles of fresh-water crabs and crayfishes, encyst and form metacercaria (*g*) which is infective 42 to 54 days. A young fluke is set free if the metacercaria is ingested with raw or poorly cooked intermediate hosts (3) (*Potamon dehaani*); it bores through the small intestine and the abdominal and thoracic cavities, remains 30 days, migrates to the pleura and bores through, gaining access to the small bronchi, where the parasites reach the adult stage and lay eggs. These eggs follow the respiratory canal and are expectorated in the sputum (*h'*). If they are swallowed, they are passed in the feces (*h*) to begin a new cycle. (Original arrangement by Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.) (*a,* After Manson; *b* and *2,* after Brumpt and Neveu-Lemaire; *c,* after Ameel in Brumpt; *3 d, e, f, g,* after Makagawa in Brumpt.)

Diagnosis.—A correct diagnosis of pulmonary paragonimiasis is made by examination of sputum, using the direct slide-cover slip preparation, and examining with a 10× ocular and 8× objective. The sputum is frequently stained with blood, and shows rust-brown flakes composed of clusters of eggs of the parasite. Differential diagnosis should be made from bronchopneumonia, tuberculosis, bronchial spirochetosis, and pleural effusion. Cysts in the lungs can be seen plainly in x-ray examination.

The eggs can be found in small numbers in the feces if the sputum is swallowed or in diarrheal stools if the parasite localizes in the intestine.

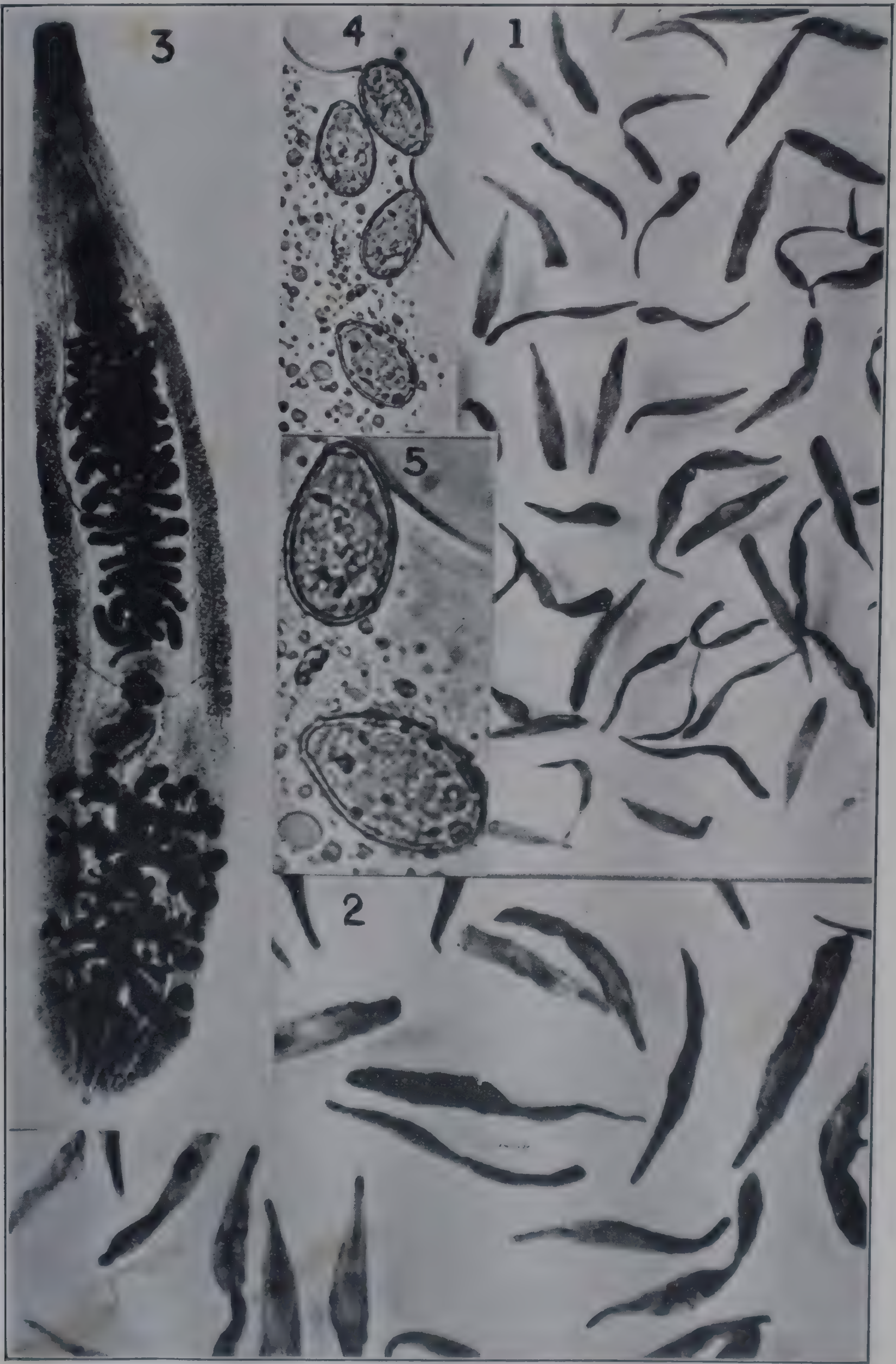


Fig. 558.—*Clonorchis sinensis.* *1,* Photograph natural size and, *2,* slightly enlarged. *3,* Photomicrograph of a specimen stained with Meyer's acid hemalum. (×12.) *4,* and *5,* Photomicrographs of eggs in bile obtained by duodenal intubation, *5,* under very high magnification. (Originals of Kourí.)

Family **OPISTHORCHIIDAE** Lühe 1908

CLONORCHIASIS

Clonorchiasis is caused by the parasite *Clonorchis sinensis*, or the Chinese liver fluke.

Clonorchis Sinensis (Cobbold 1872) Looss 1907

C. sinensis, a trematode, is a parasite of mammals which feed on fish. It is found in Japan, China, Formosa, Korea, and French Indo-China. It is prevalent in southeast China, occurs occasionally in central China, and is practically nonexistent in northern China. Emigration has not resulted in establishment of new foci of infection, since no adequate intermediate hosts have been found to exist in other countries. Many cases have been reported among the Cantonese in Cuba.

This fluke is transparent and elongated, with a thinner anterior than posterior extremity. When fixed in Formalin, some specimens are whitish, while others are brownish, more or less dark. Both forms may be present in one human liver. The parasite is very small, 2 cm. by 4 mm. The two ramified testes lie one behind the other, in the posterior portion of the parasite, behind the ovary and overlapping on each side of the intestinal cecum. A fine vas efferens or duct begins at each testis and extends forward in the dorsal plane; these unite to form a thin defferent duct, which shortly becomes thicker and forms the seminal vesicle, long and tortuous; this continues forward, still in the dorsal plane in relation to the uterine convolutions. It narrows at the ventral sucker into a fine, short ejaculatory duct directly toward the ventral surface, which opens into the genital pore in front of the ventral sucker. There is no cirrus or prostatic gland or cirrus pouch in this species.

The female genital apparatus consists of one ovary, sharply lobated, in the median line, at the junction of the third median and the posterior third of the body. It lies in front of a voluminous seminal reservoir, between this and the origin of the uterus or oötype, and is surrounded by the Mehlis gland. The vitellaria occupy the lateral portion, outside the intestinal branches, in the middle third of the parasite. These glands form a granular area which extends forward nearly to the ventral sucker, but does not quite reach it. There are two vitelloducts on each side, the anterior one long, the posterior one short, which unite to form the transverse vitelloducts. These converge toward the center to form the median vitelloduct which unites with the oviduct and the oötype where the Laurer duct and the seminal reservoir also open. The ventral sucker is smaller than the oral sucker. The intestinal branches are not ramified and end in the cecum near the posterior extremity of the body. There is a voluminous seminal receptable between the ovary and the anterior testes. The tortuous uterus extends from the oötype, near the ovary, to the genital pore, in front of the ventral sucker, and occupies the central portion of the body. The excretory canal is easily visible. It occupies a dorsal position in relation to the testes. The excretory pore appears as a protrusion in the center of the posterior extremity.

Eggs.—The egg is very small, characteristically pyriform, the posterior extremity being broader than the anterior. A prominent operculum can be seen at the narrower anterior end. It has a single yellowish-colored covering. It contains a completely developed miracidium when laid. The egg is 26 to 30 microns long by 15 to 17 microns wide.

Life Cycle.—The adult parasite lives in the bile ducts of man, dogs, cats, rats, swine, and certain wild carnivorous animals of the Far East which are the definitive hosts. The first larval forms develop within several species of mollusks or snails. The last larval stage encysts under the scales, on the skin, and in the muscles of the second intermediate hosts, which are several species of fresh-water fish. See Fig. 559.

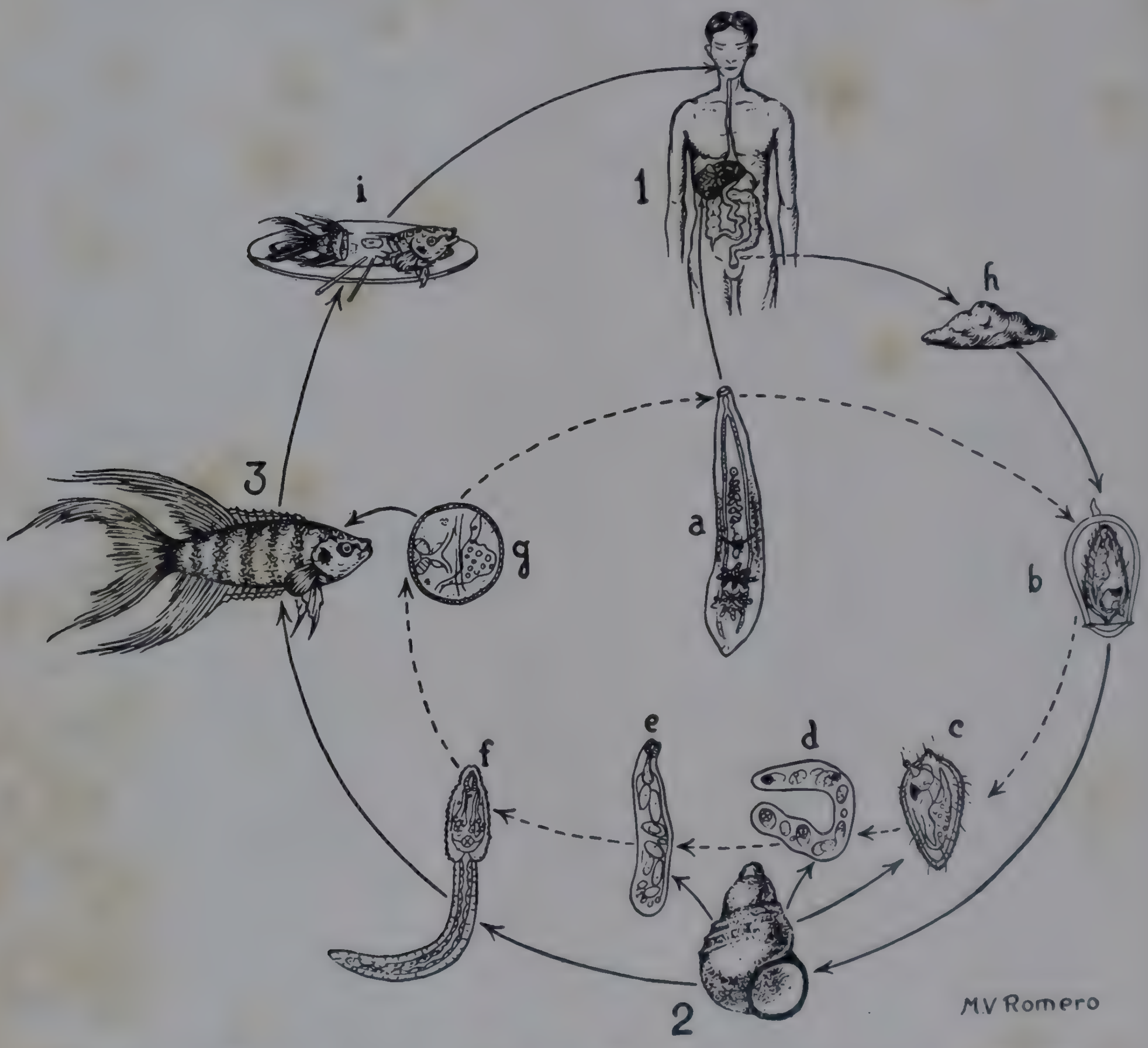
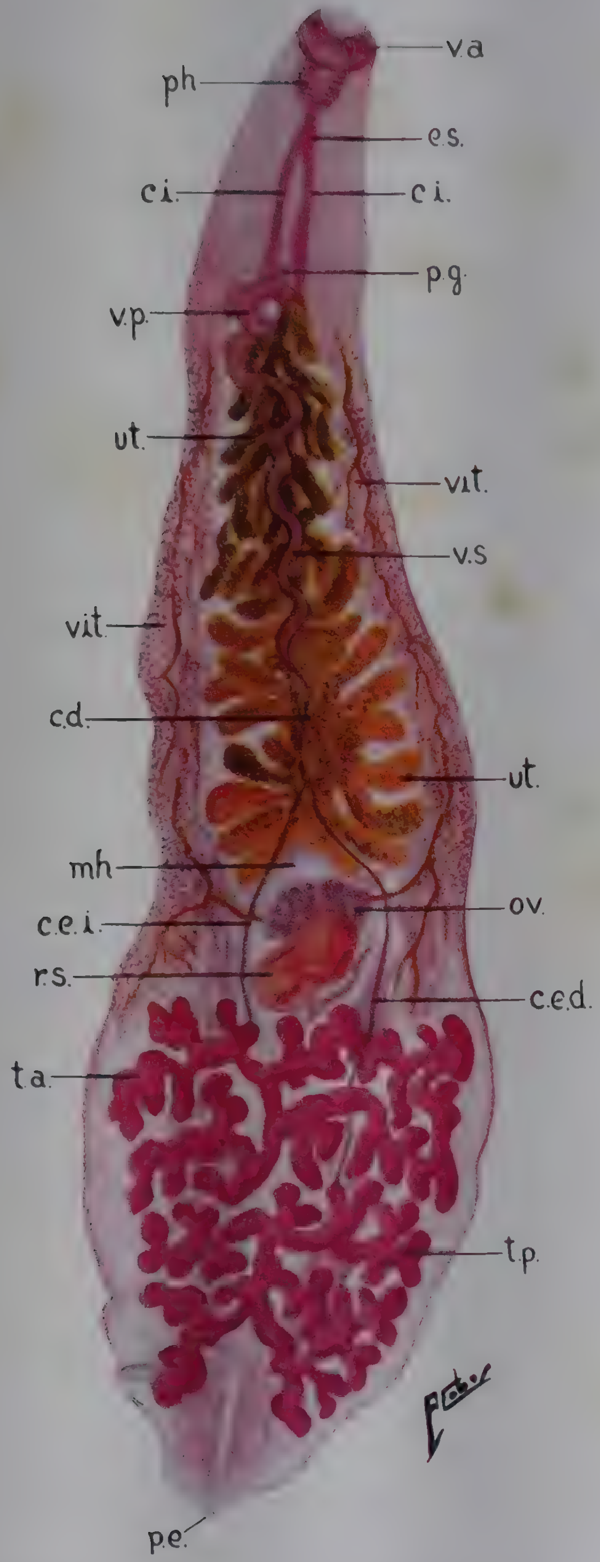


Fig. 559.—*Clonorchis sinensis.* Life cycle. The eggs (*b*), containing a miracidium, are passed in the feces (*h*), of the definitive host (*1*). If this egg (*b*) is ingested by an intermediate host (*2*), the miracidium hatches and bores through the esophagus of the mollusk (according to Faust and Khaw) to give rise to a sporocyst, rediae, and cercariae (*d, e, f*). The cercaria (*f*) leaves the snail and encysts as a metacercaria (*g*) under the scales, on the skin, and in the muscles of one of the second intermediate hosts (*3*), which are fishes. When the metacercaria (*g*) is ingested with the second intermediate hosts, it develops into an adult parasite (*a*) in the bile ducts of the definitive host (*1*). The eggs (*b*) are passed in the feces (*h*) to begin a new cycle. (Original arrangement of Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.) (*b*, In part after Faust and Khaw; *c, d, e, f, g*, after Faust and Khaw; *2*, after Germain and Neveu-Lemaire; *3*, after Neveu-Lemaire and Pellegrin. Others, original of Kourí and Basnuevo.)

This parasite may live many years in the biliary passages in man. Two cases of clonorchiasis have been reported in Panama.

Diagnosis.—In cases of digestive and hepatic disturbances, with or without fever and with eosinophilia, one should always keep in mind the possibility of clonorchiasis if the patient comes from the Far East. This disease has been found frequently in individuals who migrated from the Far East or


v.a
ph
es.
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ut.
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mh
ov.
c.e.i.
r.s.
c.e.d.
t.a.
t.p.
p.e.

Plate LII.—*Clonorchis sinensis.* (Original Figure of Kourí.) (Drawn Directly From the Microscope Stained With Carmine Hydrochloride.)

Note: *va,* anterior or oral sucker; *ph,* pharynx; *es,* esophagus; *c.i.,* intestinal ceca; *p.g.,* genital pore in front of the ventral sucker or acetabulum (*v.p.*); *ut,* uterus filled with eggs; *vit.,* vitellaria; *v.s.,* seminal vesicle; *c.d.,* vas deferens; *mh.,* Mehlis gland; *ov,* ovary; *c.e.i.,* left efferent duct; *c.e.d.,* right efferent duct; *r.s.,* seminal receptacle; *t.a.,* anterior testis; *t.p.,* posterior testis; *p.e.,* excretory pore.

China to Cuba. They have shown symptoms or clinical pictures which simulated those of malaria, bilioseptic fevers, cholecystitis, hepatic colic, and jaundice. The diagnosis must necessarily be verified by microscopic examination of the feces and of bile obtained by duodenal intubation. The eggs are so small that they might escape detection in the stool, especially in thick preparations. It is well to examine the specimen under high-power magnification. Telemann concentration method is excellent. Or use Kourí's technic for examination for fascioliasis hepatica.

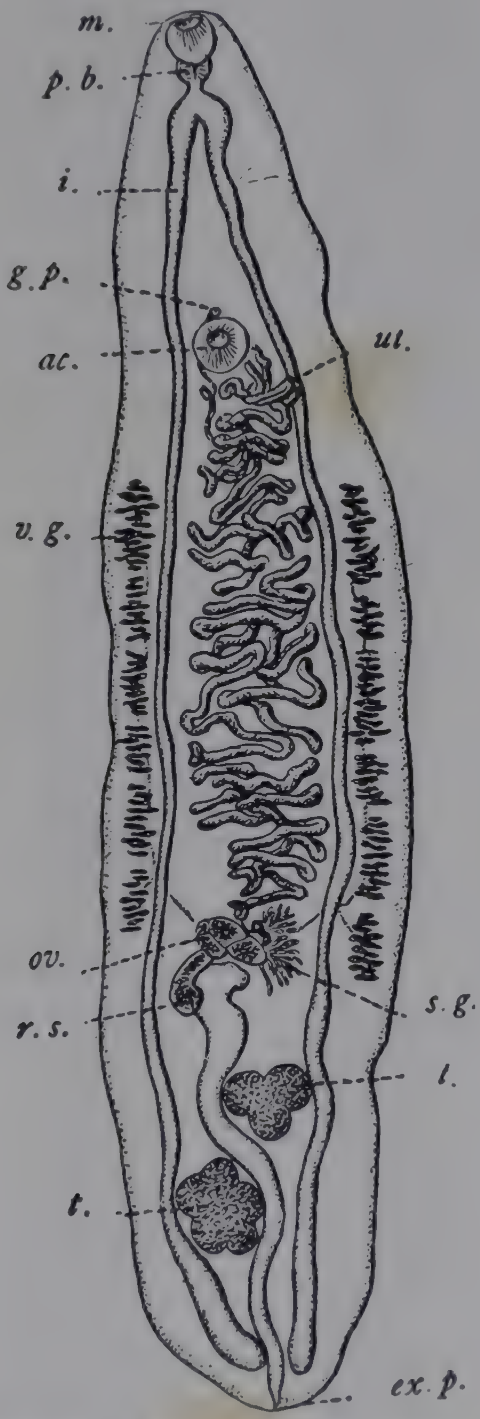


Fig. 560.—*Opisthorchis felineus:* from a cat. *m.*, Mouth; *p.b.*, pharynx; *i.*, gut; *g.p.*, genital pore; *ac.*, ventral sucker; *ut.*, uterus; *v.g.*, vitellarium; *ov.*, ovary; *s.g.*, shell gland; *r.s.*, receptaculum seminis; *t.*, testes; *ex.p.*, excretory pore. (×15.) (After Stiles and Hassall.) (From Fantham, Stephens and Theobald: The Animal Parasites of Man. Bale, Sons and Danielsson, Ltd., London.)

OPISTHORCHIASIS

Opisthorchis Felineus (Rivolta 1884) R. Blanchard 1895

Opisthorchis felineus causes a disease called the "cat liver fluke" disease. Human infection occurs as a result of eating raw or partially cooked fish infested with the metacercariae.

The parasite is found in dogs and cats in central and eastern Europe and in Siberia. It is the common parasite of dogs and cats in east and central Europe. The adult parasite is lanceolate, the anterior extremity being narrow and the posterior extremity rounded. It is 7 to 12 mm. long by 2 to 3 mm. wide. The living parasites are red or reddish-orange. There are no spines in the adult stage, but spines are visible in the sexually immature specimens. The ventral sucker is located in the anterior fifth of the body; the oral is subterminal and directed toward the ventral surface. It is followed by a bulbous pharynx, then a very short esophagus, which becomes bifurcated to form the intestinal ceca. These ceca extend almost to the posterior end of the parasite. The excretory pore is terminal. Testes are lobate and lie in the posterior fourth of the body, one on the right, one on the left of the excretory vesicle. The efferent vasa originate in the anterior portion of the testes and unite in the median region to form the vas deferens, which is directed forward, and widens to form the seminal vesicle, which ends in the ejaculatory duct, directed toward the genital atrium. There is no cirrus pouch or cirrus or prostatic glands. The ovary is small and ovoid and slightly lobate, lying along the median line slightly in front of the anterior portion of the excretory vesicle.

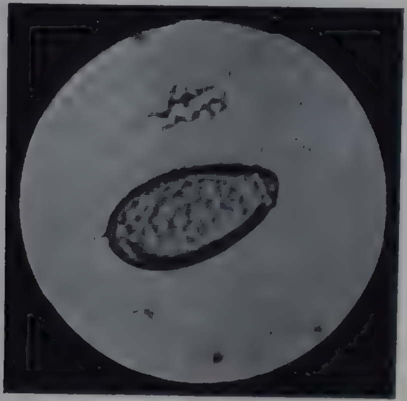


Fig. 561.—Egg of *Opisthorchis felineus* (×1,000).

Behind the ovary, to the right, is the Laurer duct; to the left is the seminal receptacle. In front of the ovary to the right is the oötype, surrounded by the Mehlis glands. The vitellaria occupy the middle third of the body, outside the intestinal cecum. The transverse vitelloducts unite to form one median vitelloduct, which joins with the oviduct before penetrating through the oötype. The uterus originates in the anterior portion of the oötype and is directed forward to form a tightly coiled tube, which ends in the vagina. The vagina opens into the genital atrium at the side of the male duct.

Eggs.—Eggs are ovoid, about 30 microns long, by 11 microns wide. When the egg is laid, the miracidium has already developed.

Life Cycle.—The egg must be ingested by the intermediate host before the miracidium leaves it. The intermediate host is *Bithynia tentaculata,* within which, near the region of the rectum, the sporocysts develop. About two months after the snail has been infected, the cercariae begin to leave. The cercariae adhere to the scales of the second intermediate host, the fish, lose their tails, then penetrate into the muscles of the fish, encysting after 24 hours. The cercariae, encysted in the muscles of the fish, require about six weeks to mature. When the definitive host ingests raw or partially cooked fish, the metacercariae leave the cyst in the stomach of the host, pass to the duodenum, and go rapidly through the ampulla of Vater to the distal bile passages, where they adhere to the biliary epithelium and grow to the adult stage in three to four weeks.

Diagnosis.—Diagnosis is established by finding the eggs in the stool, or the adult parasite.

Suborder **FASCIOLOIDEA** Stiles and Goldberger

Family **FASCIOLIDAE** Railliet 1895

FASCIOLIASIS

Fasciola Hepatica Linnaeus 1758

Fascioliasis hepatica is a cosmopolitan parasitism produced by the *Fasciola hepatica,* the sheep liver fluke. It is found in America, in Puerto Rico, Cuba, Venezuela, Argentina, and Chile; in Europe, throughout Italy, Romania, Hungary, France, Russia, and Scotland; in Asia, in China, Turkestan, Syria, Turkey; in Africa, in Algeria and French Somaliland; in Australia, in Queensland.

F. hepatica is quite commonly found among cattle. It is also found in hogs and sheep.

This fluke is flat and leaf-shaped, varying in size from 2 to 3 cm. long by 1 to 1.5 cm. at the widest portion. See Fig. 562. At the anterior extremity is a conical prolongation, on the tip of which is found the oral sucker. The ventral sucker lies from 3 to 5 mm. behind the oral sucker. It is larger and has a triangular opening, the base of which lies toward the anterior end. The body of the parasite narrows gradually. The posterior end is blunt. With a hand lens can be seen the following structures: The first two-thirds of the body has a whitish zone almost completely filled with the highly branched testes. The rest of the parasite is gray, the color imparted by the vitelline glands which are numerous. At times it is dark brown, the color coming from the digested blood in the blind intestinal branches which are numerous throughout this region. The rosette-shaped uterus is filled with eggs; it is a distinct yellowish mass continuing cephalad the clear zone described above. There is a rounded area corresponding to the ventral sucker lying before the uterine mass or rosette; in front of this are the cirrus sac and the small genital pore. The short esophagus, muscular pharyngeal blub, and the oral sucker are anterior to the aforementioned structures, in the order described. A circular zone, the cochlear or shell gland, is clearly visible directly behind the undulations of the uterus. Transverse vitelline ducts, vitelline ampulla, both located posterior to the cochlear gland, and the branched ovary at the right of the median line in front of the ramification of the anterior testes can also be seen. The short esophagus divides into trunks. The cirrus sac lies behind the fork made by the trunks. From its convex surface each trunk sends ramifications which extend toward the anterior conical prolongation of the parasite and is called the cephalic cone. The two trunks turn back on each side of the median line, then send out external ramifications which extend toward the borders of the body.

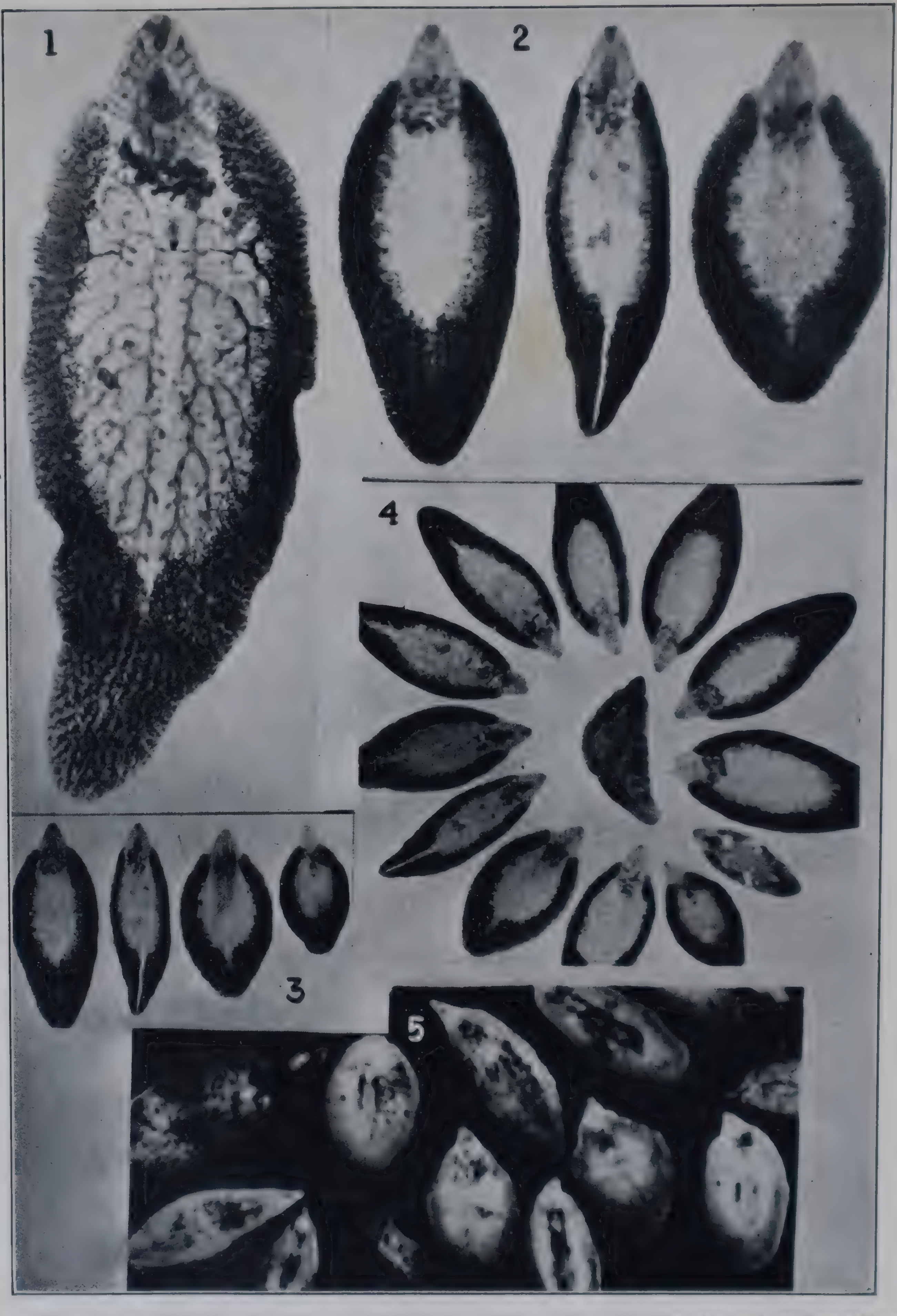


Fig. 562.—*Fasciola hepatica*, characteristic shape with anterior conical prolongations. The white central oval zone is occupied by the testes, *1*, *2*, *3*, *4*. The dark zone contains the vitelline glands. *1*, The white central zone is crossed by the two intestinal branches with lateral external ramifications. The trunks and their branches end in blind pouches. Proceeding from the front toward the back of the anterior conical prolongations, note the anterior (oral) sucker, pharynx, the arch formed by the intestinal trunks, the cirrus sac, ventral sucker, and the uterus.

1, Original of Vigueras and Moreno; *2* to *5*, originals of Kourí, Arenas, and Basnuevo; *1* to *4*, photographs illuminated from below; *5*, dark background, illuminated from above; *3* to *5*, natural size; *1* and *2*, various magnifications.

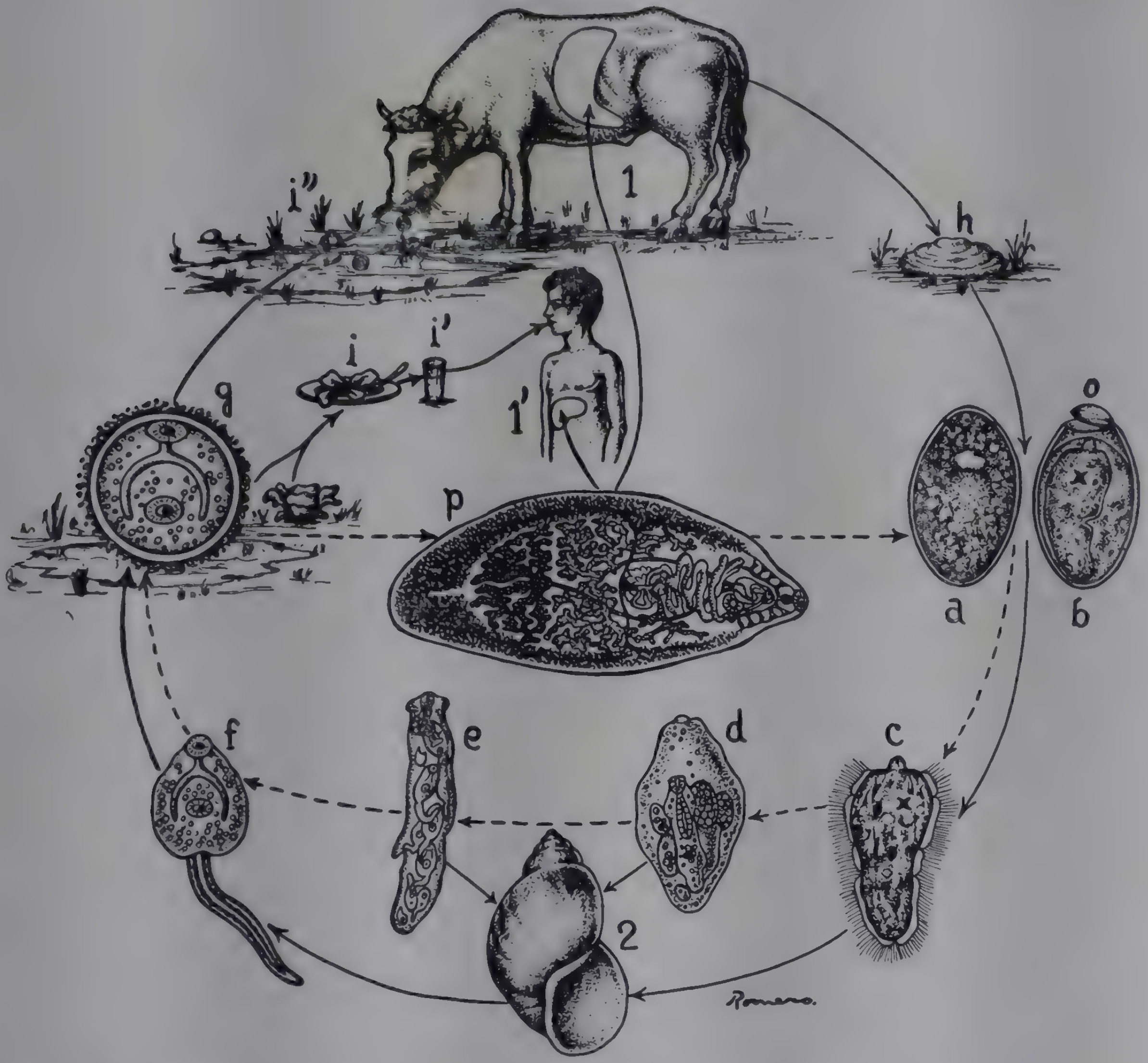


Fig. 563.—*Fasciola hepatica.* Life cycle. The adult parasite (*p*) lives in the bile ducts of the definitive host (cattle, *1;* man, *1'*). The eggs (*a*) are passed in the feces (*h*); after reaching the water they become embryonated (*b*), the miracidium (*c*) hatches through the operculum (*o*), and swim freely in the water until finding the intermediate host (*2*) (*Lymnea cubensis* and *Physa cubensis* in Cuba), where the sporocysts (*d*), rediae (*e*), and cercariae (*f*) develop. The cercaria (*f*) leaves the snail and becomes encysted in the water either freely or attached to water plants, developing into the metacercaria (*g*) which is the infective stage for the definitive host (*1, 1'*). When the definitive host ingests the metacercaria with water or vegetables (*i, i', i"*) it reaches the intestine, the cystic cover dissolves, and frees a young fluke; this bores through the intestinal wall and reaches the liver surface through the peritoneum; when boring through the fibrous capsule and the liver parenchyma, the parasite produces necrosis; in the bile duct it becomes an adult hermaphroditic parasite and lays eggs which are passed in the feces (*h*), beginning a new cycle. (Original arrangement by Kourí and Basnuevo in Lecciones de Parasitología y Medicina Tropical.) (*d, e, f, g,* After Leuckart; others, original of Kourí and Basnuevo.)

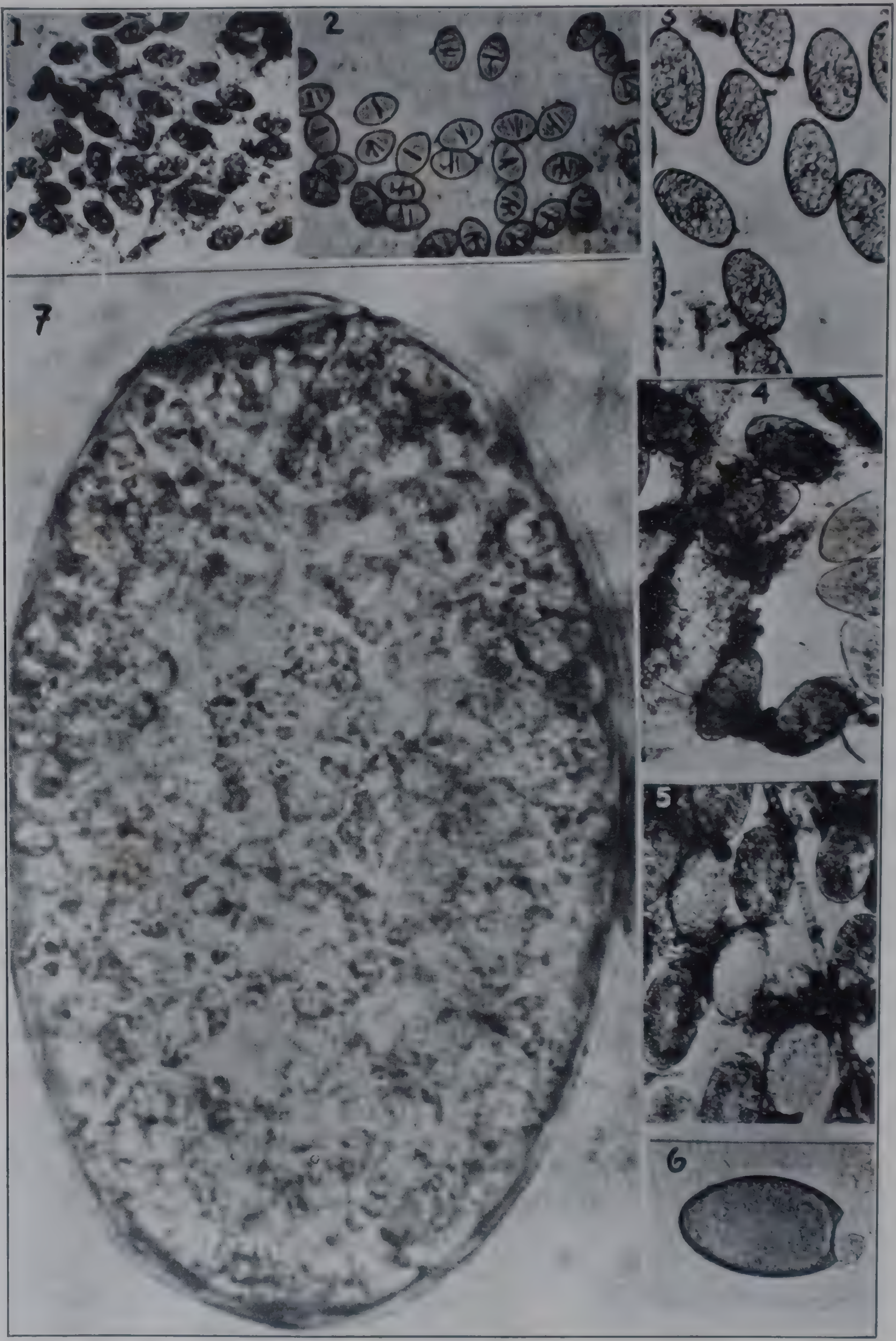


Fig. 564.—*Fasciola hepatica.* (Originals of Kourí. Photomicrographs of eggs in human bile obtained by duodenal intubation.)

1, Groups of eggs, surrounded by yellow mucoid flecks suspended in the bile (oviferous flakes).

2. Eggs in bile mucus. The transverse folds are produced by pressure of the cover glass after the specimen dries.

3 to *5*. Eggs surrounded by yellow bile flakes, higher magnification than in *1*.

6. Egg in bile with the operculum elevated by pressure upon the cover glass.

7. Egg in bile. The small operculum can be seen clearly in the upper pole. The contents are composed of numerous polygonal vitelline cells, granular, and with small nuclei

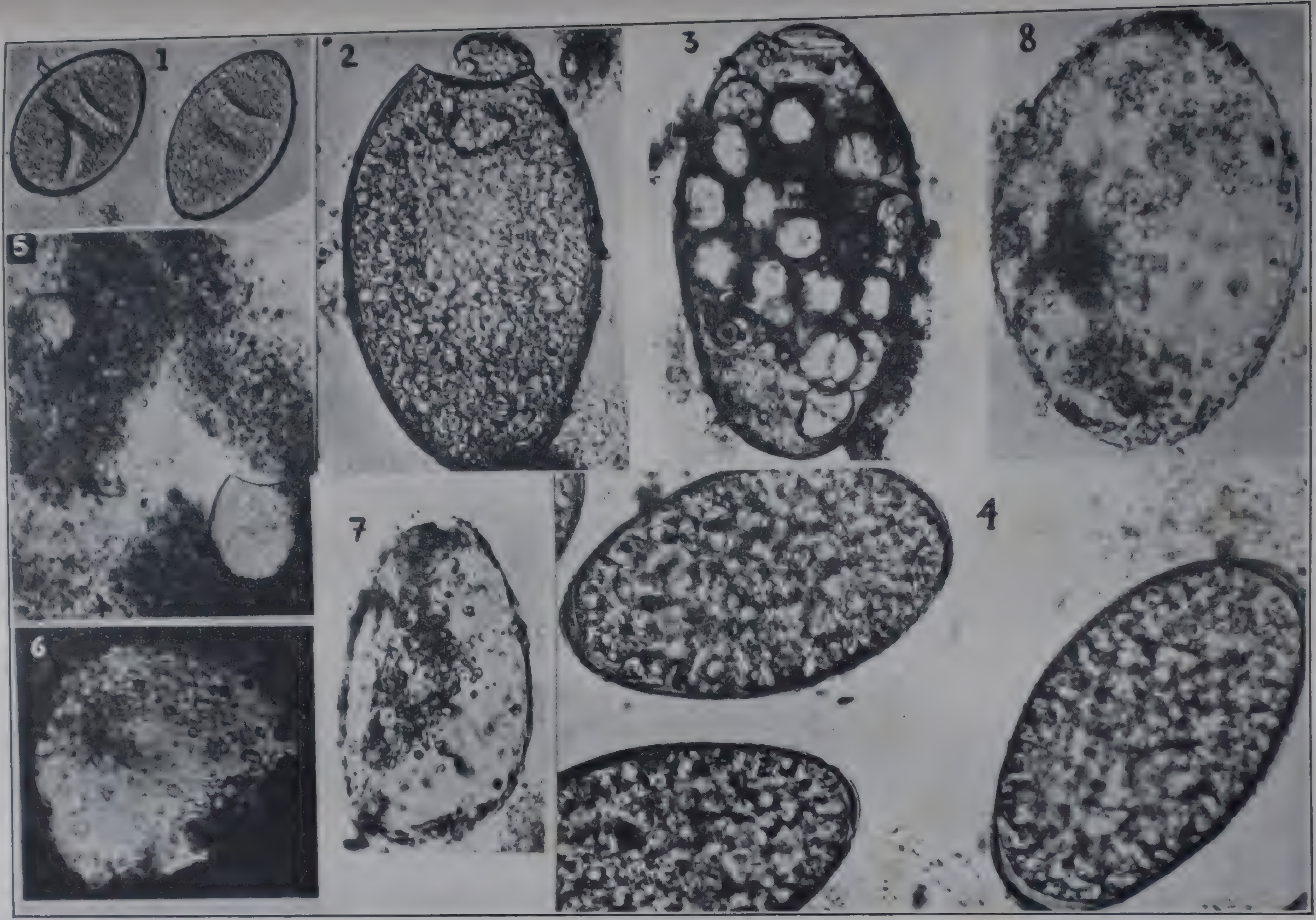


Fig. 565.—*Fasciola hepatica.* (Originals of Kourí, Basnuevo, and Arenas.) Photomicrographs of eggs in human bile obtained through duodenal intubation.

1, Eggs with transverse folds caused by pressure of the cover glass when the specimen undergoes drying. *2*, Egg with elevated operculum and granular protoplasm. *3*, Egg with spherical refractive cells and slightly elevated operculum; derived from freshly removed bile. *4*, Typical eggs in human bile. *5* to *8*, Eggs in various stages of atrophy and degeneration as the result of the action of small doses of emetine hydrochloride, a powerful fasciolicide. Human bile obtained through duodenal intubation. Case under treatment; specimens obtained after the initial doses of emetine.

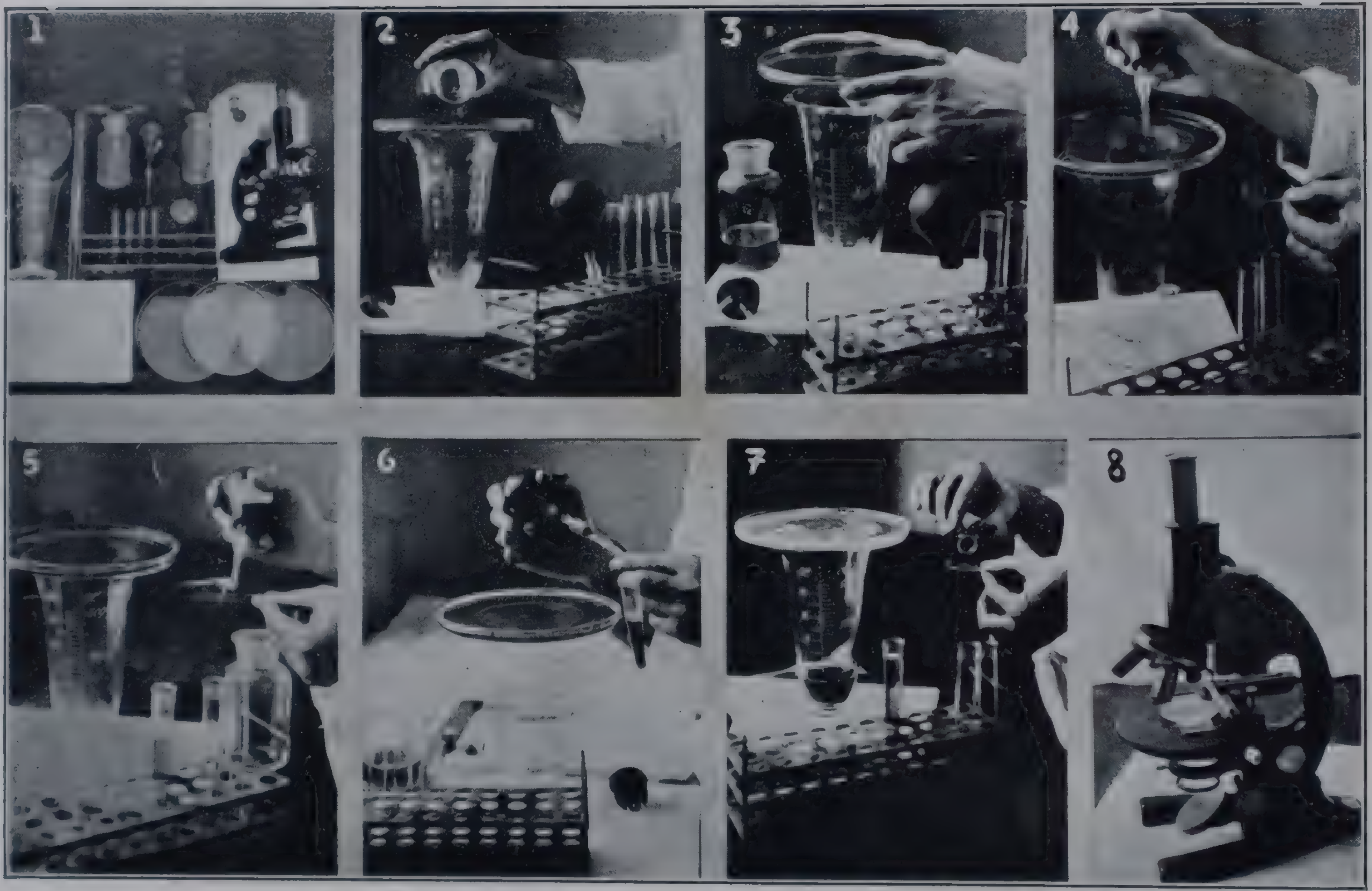


Fig. 566.—*Fasciola hepatica.* Kourí's method of examination of bile obtained through duodenal intubation for the diagnosis of biliary and duodenojejunal parasitism, particularly by *Fasciola hepatica.*

1, Materials needed: graduated beaker (500 to 1,000 c.c.), Petri dishes, 20 cm. in diameter, brick, mold, centrifuge tubes, pipettes, watch glasses, slides and cover glasses, microscope, substage lamp, black paper, white paper. *2*, Step 1: Pour bile into Petri dish on the beaker which is in turn placed upon a piece of white paper. *3*, Step 2: Observe macroscopic characteristics of bile, paying particular attention to the appearance of the suspended mucus. Move the Petri dish alternately over a black and a white background. *4*, Step 3: Brown or yellow mucus contains numerous eggs; white mucus contains none. Use a short pipette with a large bore and with a large bulb; suck up the suspected mucus flakes, transfer to a watch glass or slide. *5*, Examine microscopically. The eggs are found in greater numbers in the center of the watch glass. *6*, Step 4: Transfer all the flakes to the centrifuge tube. *7*, Step 5: Examine microscopically in a watch glass all the centrifuged flakes after washing them and decanting the supernatant fluid. *8*, Step 6: Place the watch glass with the flakes on a slide on the mechanical stage. Use low power (3X ocular, 3 objective, Zeiss). With binocular dissecting microscope examination is made directly in the Petri dish placed on the mechanical stage. This technic affords a rapid and accurate diagnostic method for fascioliasis hepatica. Examination of oviferous flakes (Kourí) usually provides sufficient evidence of infestation. This technic is very useful in clonorchiasis, lambliasis, and strongyloidiasis because of the frequency of negative results in examination of the feces. It is valuable in necatoriasis, ancylostomiasis, and biliary ascaridiasis.

Eggs.—The egg is one of the largest helminthic eggs of importance in human parasitology. It is ovoid, about 150 microns long and 80 microns wide in its widest portions. It has a yellow cover or shell, with a relatively small operculum. The contents consist of a fertilized ovule, often near one of the poles, surrounded by numerous polygonal vitelline cells.

Life Cycle.—The eggs are excreted in the feces. In tropical countries, they embryonate in water in about 2 or 3 weeks at a temperature of about 20° C. (68° F.). Embryonation of the eggs results in ciliated embryos which escape through the opercula, and swim freely in the water. The complete life cycle is given in Fig. 563.

Development of *F. hepatica* in the bile ducts may cause a series of lesions known as hepatic distomiasis, or, more specifically, hepatic fascioliasis. During the period of invasion, there is a febrile eosinophilia, and acute abdominal symptoms with hepatitis. The eggs do not appear for three months. The majority of patients studied have given a long history of gastrointestinal, hepatobiliary, nervous, and general disorders. The real cause of this disease is not known until the parasite is discovered in the patient.

In Fig. 566 is found Kourí's method of examination of bile obtained through duodenal intubation.

Diagnosis.—Examination of the feces may show the characteristic eggs of the parasite. Direct examination of feces and concentration methods for eggs should be used. One or more negative coprologic examinations are not sufficient proof to deny, in an absolute way, the possibility of distomiasis by *F. hepatica.* For further proof, one must resort to parasitologic examination of the bile obtained through duodenal intubation. See Fig. 566.

Eosinophilia is quite frequent. Intradermal reaction, precipitin reaction, and complement fixation reaction are indirect methods which may be of some value. The antigen used consists of extracts of *F. hepatica.*

DISEASES CAUSED BY INTESTINAL FLUKES

None of the diseases caused by intestinal flukes has been reported in the Western Hemisphere.

FASCIOLOPSIASIS

Fasciolopsis Buski (Lankester 1857) Odhner 1902

Fasciolopsis buski, also called the "giant intestinal fluke," is found in central and southern China, Formosa, Siam, Borneo, Sumatra, Assam, and Bengal. It is a common parasite of man and pigs.

The adult worm, which attaches itself to the walls of the duodenum and jejunum, is a large, fleshy parasite, at times oval, but usually elongated and ovoid. It varies in size, from 20 to 75 mm. long, 8 to 20 mm. wide, and 0.5 to 3 mm. thick. The surface of the parasite is covered with spines. The oral sucker is about 0.5 mm. in diameter, while the ventral sucker is approximately 3 mm. in diameter. The intestinal ceca are not ramified. The testes have many branches and lie one behind the other in the posterior half of the parasite. The cirrus pouch is elongated, with a seminal vesicle, sinuous in the posterior portion. Prostatic glands surround the ejaculatory duct. It has a muscular type cirrus canal covered with spines, and a pre-cirrus canal which opens into the general atrium, which is immediately in front of the ventral sucker. The oötype is found in the center of the parasite and is surrounded by the Mehlis gland. The ovary is ramified and lies to the right of the median line. The Laurer canal is small, a seminal receptacle not having been described. The vitelline glands are formed by small, granular follicles, which extend into the

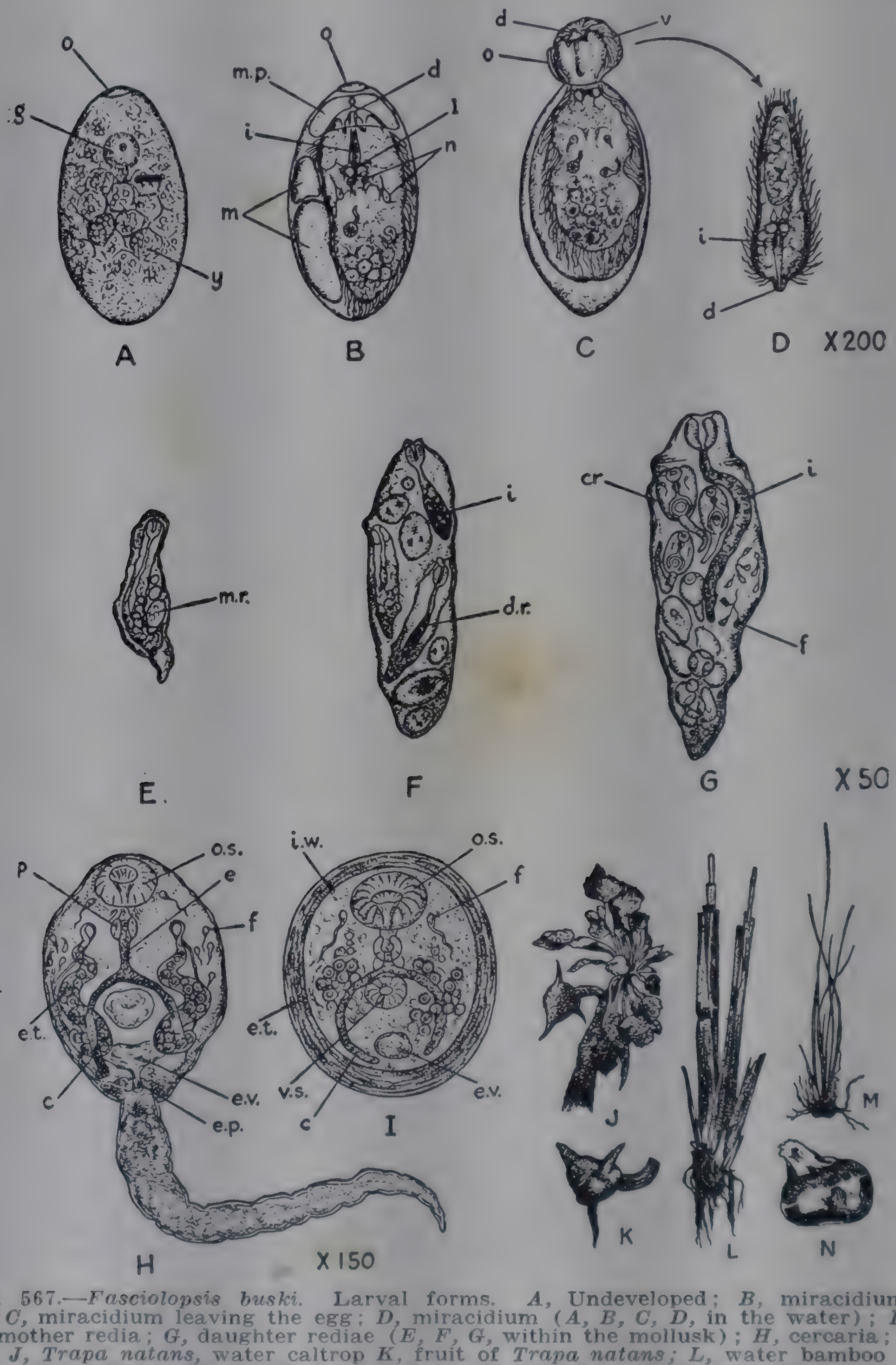
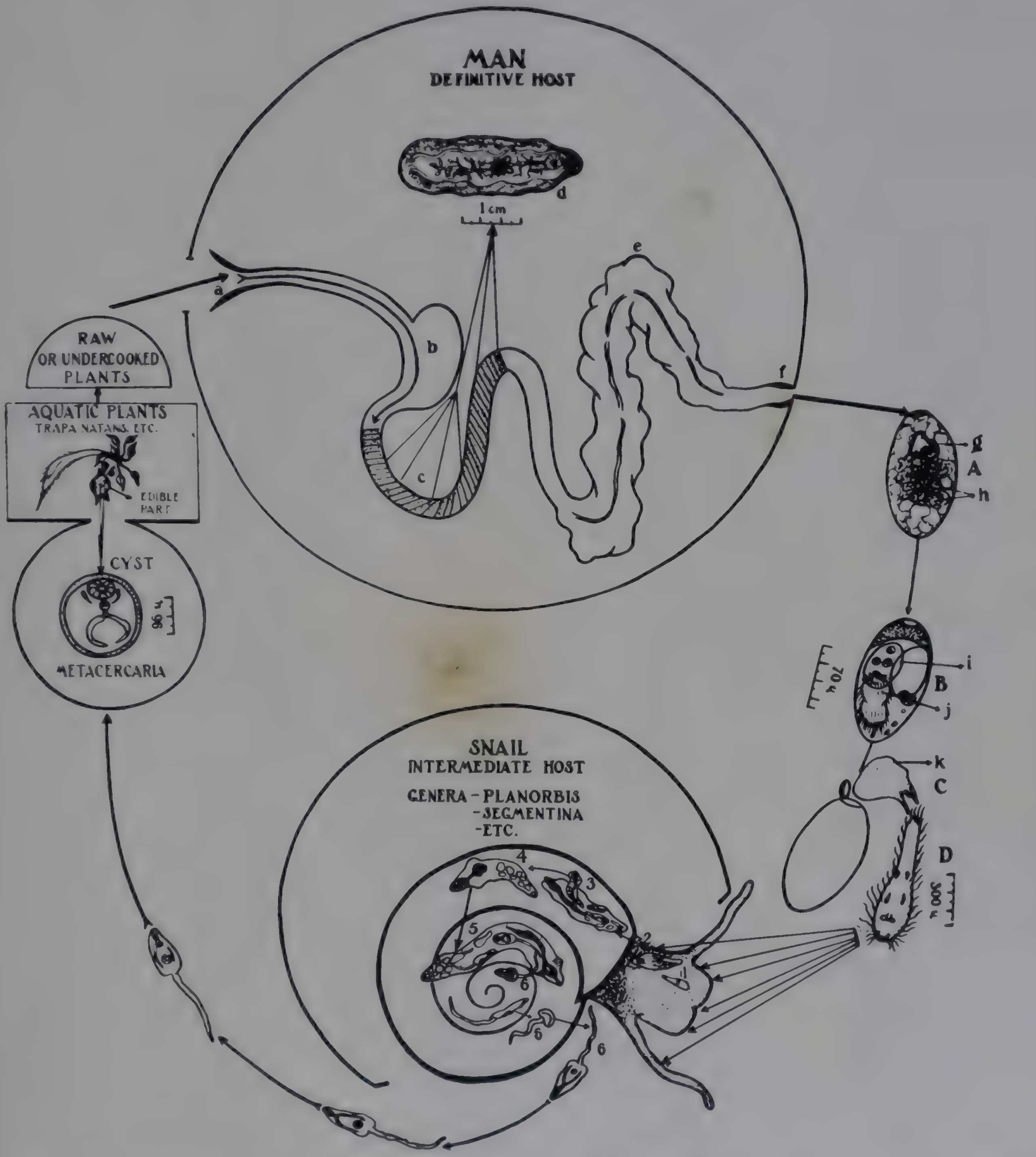


Fig. 567.—*Fasciolopsis buski.* Larval forms. *A*, Undeveloped; *B*, miracidium within the egg; *C*, miracidium leaving the egg; *D*, miracidium (*A, B, C, D*, in the water); *E*, sporocyst; *F*, mother redia; *G*, daughter rediae (*E, F, G*, within the mollusk); *H*, cercaria; *I*, metacercaria; *J*, *Trapa natans*, water caltrop *K*, fruit of *Trapa natans*; *L*, water bamboo (*Zizania aquatica*); *M*, water chestnut (*Eliocharis tuberosa*); *N*, tuber of the water chestnut (*H* to *N* in the water); *c*, ceca; *cr*, cercaria; *d*, protuberance from the anterior extremity of the miracidium; *d.r.*, daughter redia; *e*, esophagus; *e.p.*, excretory pore; *e.t.*, excretory concretions; *e.v.*, excretory vesicle; *f*, flame cell; *g*, germinal area; *i*, primitive digestive tract; *i.w.*, inner cyst wall; *l*, optic lens; *m*, oil masses; *m.p.*, mucoid plug; *m.r.*, germ mass from which mother redia develops; *n*, neurone; *o*, operculum; *o.s.*, oral sucker; *p*, pharynx; *v*, vitelline membrane; *v.s.*, ventral sucker; *y*, yolk, *A* to *J* redrawn from Barlow, 1925. *K* to *N* redrawn from Wu, 1937, by Belding in Textbook of Clinical Parasitology. (From Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

lateral fields, from the ventral sucker to the caudal extremity. The uterus follows a tortuous course between the öotype and the metraderm, which can be found immediately within the genital pore.

Eggs.—The eggs are quite similar to those of *F. hepatica.* They are 130 to 140 microns long by 80 to 85 microns wide, and are provided with an operculum. They do not contain a developed miracidium when first laid.


MAN
DEFINITIVE HOST
d
1 cm
e
a
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c
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g
A
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i
B
j
70 µ
k
C
D
300 µ
RAW
OR UNDERCOOKED
PLANTS
AQUATIC PLANTS
TRAPA NATANS, ETC.
EDIBLE
PART
CYST
96 µ
METACERCARIA
SNAIL
INTERMEDIATE HOST
GENERA - PLANORBIS
- SEGMENTINA
- ETC.
1
2
3
4
5
6
6
6

Fig. 568.—Life cycle of *Fasciolopsis buski.* (Chart planned and drawn by Dr. Alfonso Bonilla Naar, of Bogotá, Colombia, and published through his courtesy by permission of copyright owner, General Biological Supply House, Chicago, Illinois.)

Life Cycle.—The eggs are passed in the feces of the definitive host. It requires 3 to 7 weeks at 26° to 32° C. (79° to 89° F.) for the miracidium to develop. This miracidium forces open the operculum and swims freely until it finds an appropriate host, a snail. It penetrates into the soft tissues of the snail, and in a few weeks gives rise to sporocysts, rediae, and cercariae. The molluscan hosts in China and Formosa are *Planorbis caenosus, Segmentina nitidella, S. calathus,* and others.

The cercariae are quite similar to those of *F. hepatica.* In endemic regions, cercariae encyst in the roots and bulbs of certain aquatic plants. Man is infected by husking the infected plants between the teeth and lips, before eating the contents. The metacercariae leave the cyst in the duodenum, and cling to the walls of the intestine, where they grow to the adult stage in about 3 months. Human infection is associated with eating certain uncooked aquatic plants cultivated in endemic areas where the appropriate host is abundant.

The life cycle is further exemplified in Fig. 568.

Diagnosis.—Diagnosis is based on finding the eggs in the stools of parasitized individuals.

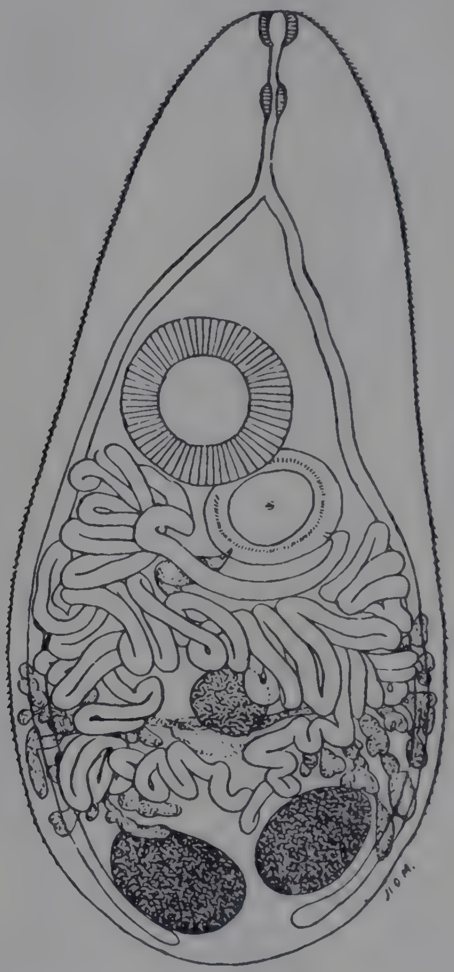

Fig. 569.—*Heterophyes heterophyes,* after Mönnig; adult, ventral face. Note that the posterior extremity is thicker than the anterior. Note the large ventral sucker (acetabulum), and, immediately behind it, the characteristic genital sucker. The two testes are oval, posterior, and horizontal. (×60.) (Kourí and Basnuevo: Lecciones de Parasitología y Medicina Tropical.)

HETEROPHYIASIS

Heterophyes Heterophyes (von Siebold 1852) Cobbold 1886

The worm is very small, 1.5 mm. long and 0.5 mm. wide.

Eggs are small, brownish, and have a thick shell; they are about 26 to 30 microns by 15 to 17 microns.

This fluke is a habitual parasite of dogs, cats, rabbits. It has two intermediate hosts, a salt-water mollusk, and fishes of the family Mugilidae, in the muscles of which the metacercariae infective for man encyst. Man is infected by eating fish contaminated with the parasite.

METAGONIMIASIS

Metagonimiasis is parasitism produced by *Metagonimus yokogawai*. It is found in the Far East, in Northern Siberia, the Balkan States, and Spain.

Metagonimus Yokogawai Katsurada 1913

The adult parasite is very small, 1 to 2.5 mm. long by 0.4 to 0.75 mm. wide. The body is pyriform. The posterior extremity is rounded; the anterior extremity is narrowed and covered with scales. The ventral sucker lies to one side of the median line. The oral sucker is continued by the esophagus which is divided into a pair of intestinal ceca which end in the posterior region of the body. The excretory vesicle is tubular and has two prolongations. The testes lie in the posterior extremity. They are partially or completely lobulated. The ovary is globular, approximately the same size as the testes, and lies along the median line, at the junction of the middle third with the posterior third of

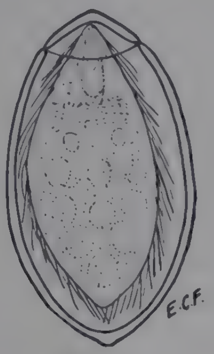

Fig. 570.—Egg of *Heterophyes heterophyes*. Camera lucida drawing. (×1,120.) (Original figure in Human Helminthology by Faust, 1939. Courtesy Lea & Febiger, Philadelphia.)

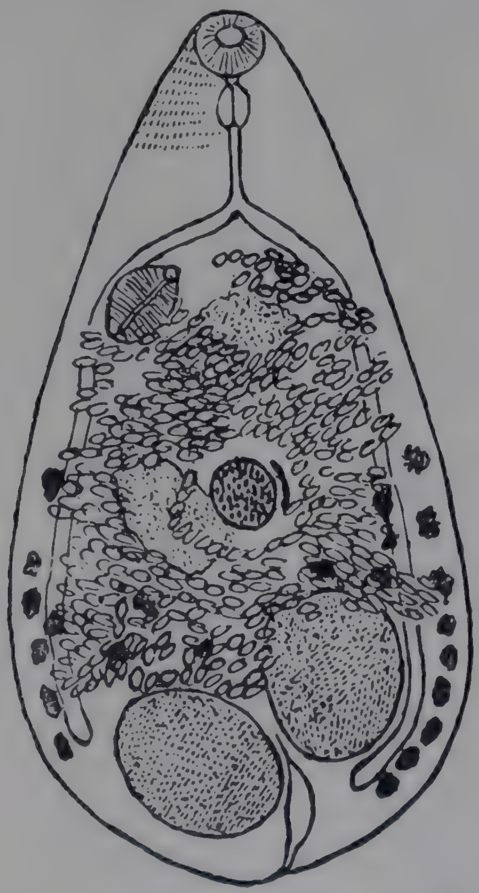

Fig. 571.—*Metagonimus yokogawai*. (×50.) The spines are shown only over a small part of the skin. (After Leiper.) (From Fantham, Stephens and Theobald: The Animal Parasites of Man. Bale, Sons and Danielsson, Ltd., London.)

the body. Toward the back and slightly to the left are the seminal receptacle and the Laurer canal. The oötype and the Mehlis gland are at the left of the ovary. The vitelline glands form a fan-shaped cluster on each side of the parasite. The vitelloducts are directed toward the center of the parasite, forming a median vitelloduct behind the oötype, which it penetrates with the oviduct. The uterus follows a sinuous course until it opens, with the ejaculatory duct, into the genital atrium.

Eggs.—The eggs are brownish-yellow, operculated, ovoid, and 28 microns long by 17 microns wide. When first laid, the eggs contain a completely developed miracidium.

Life Cycle.—The first intermediate hosts are the *Melania libertina* and the *M. ebenina,* within which the sporocysts, rediae, and cercariae are formed. These leave the snail and swim freely in the water until they reach an appropriate host. They attack the host and bore under the scales into the muscles of the fish, the second intermediate hosts. The most common intermediate hosts in Japan are the fresh-water fishes. The larvae encyst in the muscles and grow according to the quantity of nutritive material available and the length of time they have to spend within the fish.

Man is infected by eating raw contaminated fish. In the duodenum, the young distome breaks through the cystogenic membrane which protects it as it passes through the stomach, and adheres to the intestinal mucosa, where it grows into an adult parasite.

Diagnosis.—Diagnosis is based on finding the eggs in the stools, where they must be differentiated from those of *Clonorchis sinensis* and *Opisthorchis felineus.*

DISEASES CAUSED BY ARTHROPODA

Bacteria-Carrying Arthropoda

Arthropoda may carry bacteria and may also serve as intermediate hosts for certain animal parasites. There are a number of mites which may attack man by burrowing into the skin, thus causing inflammation and itching. These are known as mites, red bugs, chiggers, etc. There are many ticks which produce irritating lesions on the skin, but do not produce disease. There are certain ticks, however, that are intermediate hosts for transmission of diseases like relapsing fever and Rocky Mountain spotted fever. Rocky Mountain fever is communicated by the wood tick, *Dermacentor andersoni.*

Certain parasitic insects are found in practice: lice, fleas, and the larvae of certain flies. Fleas have favorite hosts, but may fly to other species. The best-known human flea is called the *Pulex irritans.* A dog flea, *Ctenocephalus canis,* also attacks man at times. The rat fleas, *Xenopsylla cheopis* and *Ceratophyllus fasciatus,* are important because they are concerned with the transmission of plague.

Sarcoptes Scabiei Var. Hominis

A full discussion of the life cycle of *Sarcoptes scabiei* var. *hominis* has been given by Lieutenant Commander Eugene A. Hand (MC) U.S.N.R.*

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*U. S. Nav. M. Bull. 46: 834, 1946. Permission has been given by the Navy Department, through Captain Louis H. Roddis (MC) U.S.N., Editor U. S. Naval Medical Bulletin, to use the facts and figures presented here.

The first description of *Sarcoptes scabiei* var. *hominis* was given by Bonomo[1] in 1687. This author verified the findings of Friedman[2] that the Acarus could easily be demonstrated by the scrape and smear method and also that the diagnosis could be made from finding the eggs, filled or empty, skeletal parts of the Acarus, the larvae, and the scybala as well as from the adult Acarus. Mary Hopper[3] found this scrape and smear method also by accident and has used it at the Cornell University Medical School. The photomicrographs (Figs. 575 to 583) of the adult female, the hexapod larvae, the Acarus eggs in various stages of hatching, empty Acarus eggs, the Acarus burrow, skeletal parts of the Acarus, and scybala are shown to aid in identifying them.

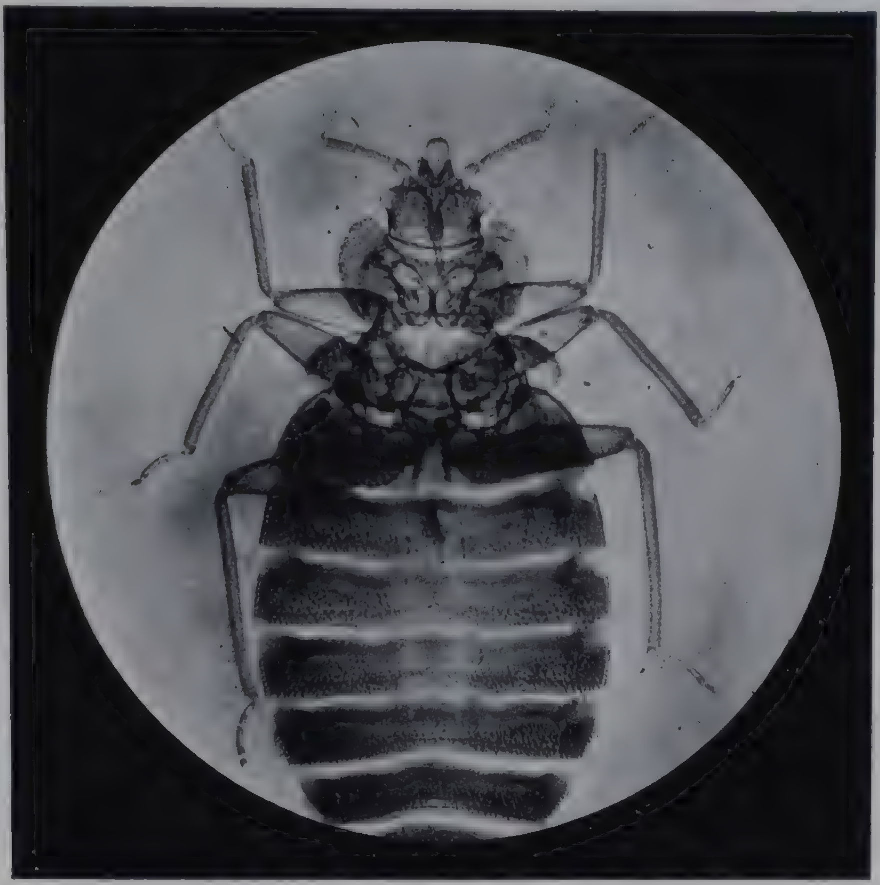

Fig. 572.—Bedbug, *Cimex* (×30).

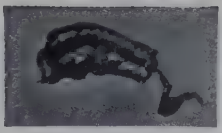

Fig. 573.

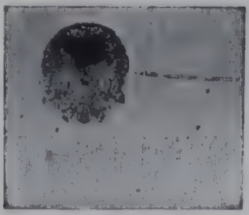

Fig. 574.

Fig. 573.—Demodex mite (×80).
Fig. 574.—Mange mite, *Sarcoptes scabiei* (×80).

Technic of Finding the Sarcoptes Scabiei Organism and Its Parts.—Hand states that it is best to make a careful search over the parts affected to pick out lesions that will most likely give a positive diagnosis. Ordinary rules of cleanliness and asepsis should be followed. For lesions on the fingers, on the inter-

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[1] Lane, J. E.: Bonomo's Letter to Redi; Important Document in History of Scabies, Arch. Dermat. & Syph. 18: 1, Sept., 1928.

[2] Pennsylvania M. J. 47: 39, Oct., 1943.

[3] Personal communication.

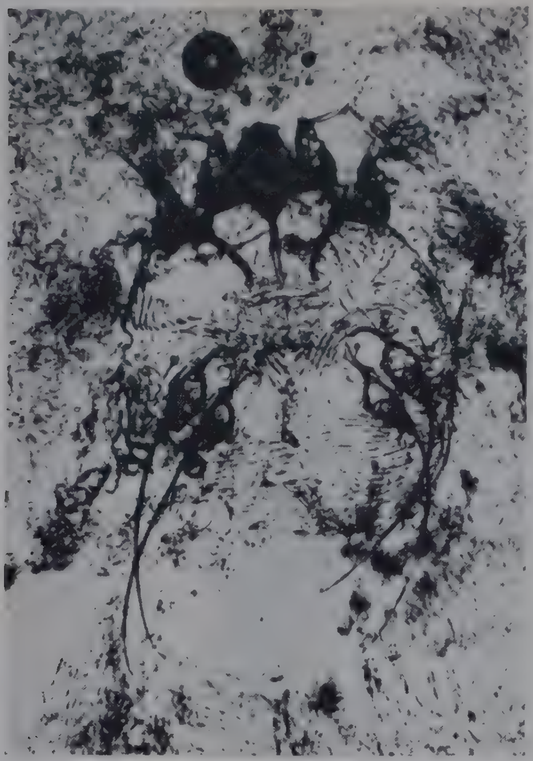

Fig. 575.

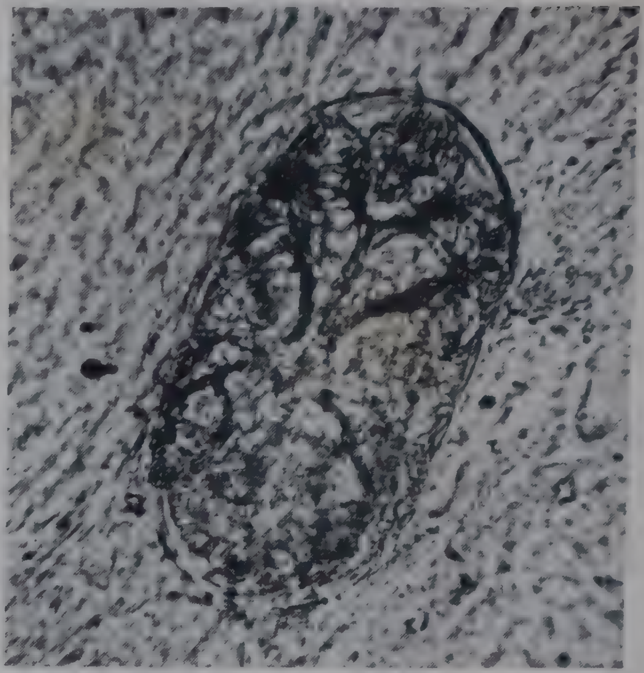

Fig. 576.

Fig. 575.—Diagnosis of infestation by the *Sarcoptes scabiei* var. *hominis* by the slice, scrape, and smear method. Ventral view female adult Acarus. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in the U. S. Naval Medical Bulletin **46: 834-844, June, 1946, by courtesy of the author and the publisher.)**

Fig. 576.—Diagnosis of infestation with the *Sarcoptes scabiei* var. *hominis* by the slice, scrape, and smear method. Acarus egg with embryo. High power. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in U. S. Nav. M. Bull. **46: 834-844, June, 1946, by courtesy of the author and the publisher.)**

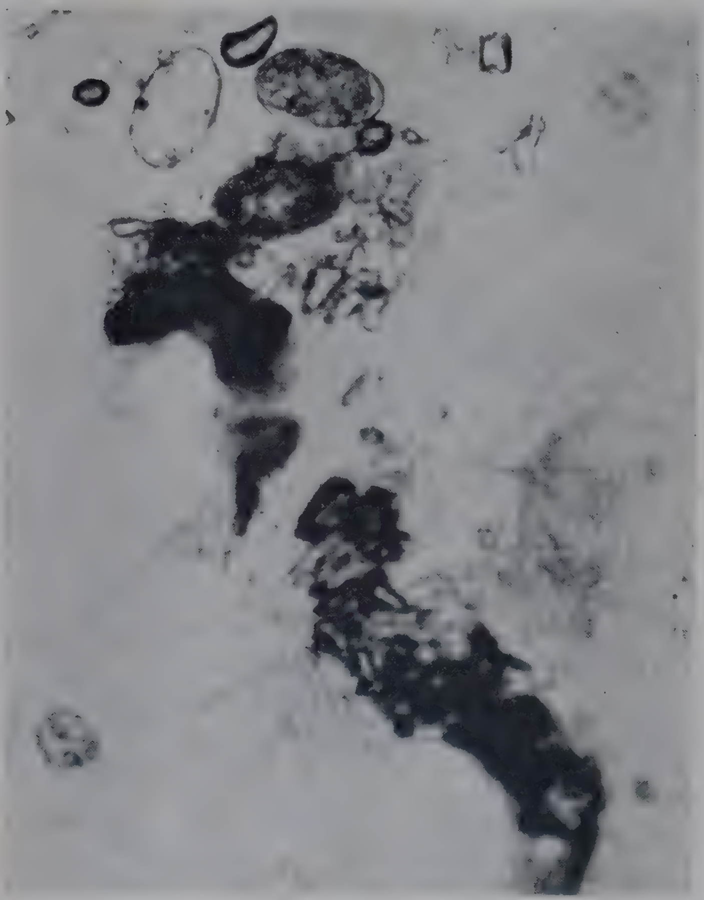

Fig. 577.

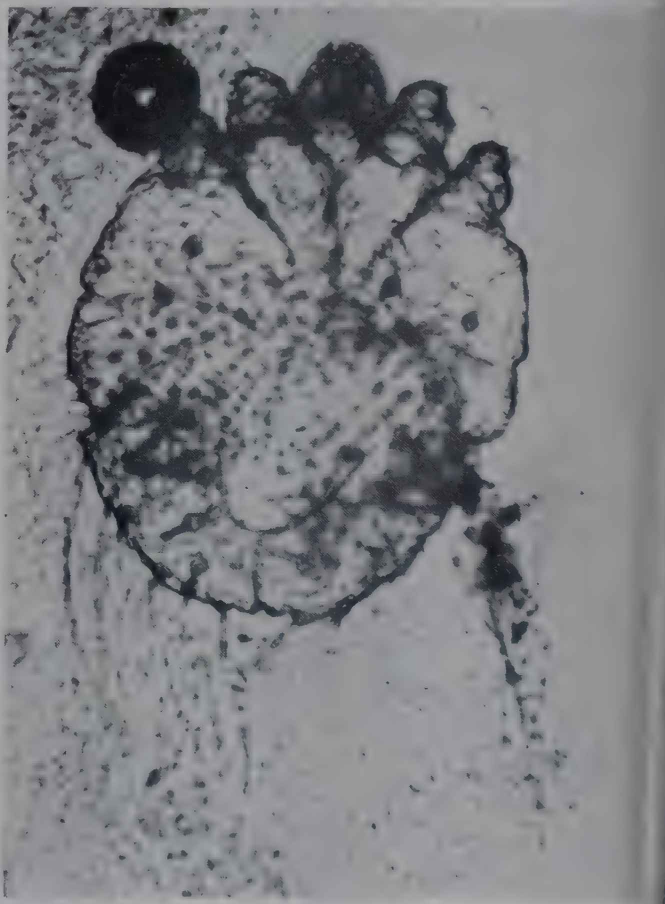

Fig. 578.

Fig. 577.—Diagnosis of infestation with the *Sarcoptes scabiei* var. *hominis* by the slice, scrape, and smear method. Burrow showing eggs and Acarus feces or scybala. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in the U. S. Nav. M. Bull. **46: 834, 1946, by courtesy of the author and the publisher.)**

Fig. 578.—Diagnosis of infestation with the *Sarcoptes scabiei* var. *hominis* by the slice, scrape, and smear method. High power of adult female. Acarus showing egg in oviduct. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) (USNR, in U. S. Nav. M. Bull. **46: 834, 1946, by courtesy of the author and the publisher.)**

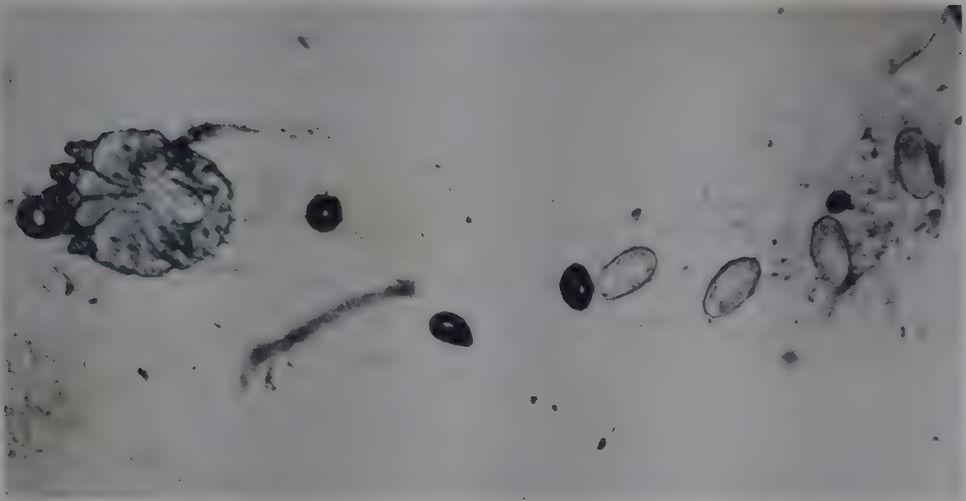

Fig. 579.—Diagnosis of infestation with the *Sarcoptes scabiei* var. ***hominis*** by the slice, scrape, and smear method. Acarus burrow with adult, female Acarus and Acarus eggs. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in U. S. Nav. M. Bull. **46**: 834, 1946, by courtesy of the author and the publisher.)

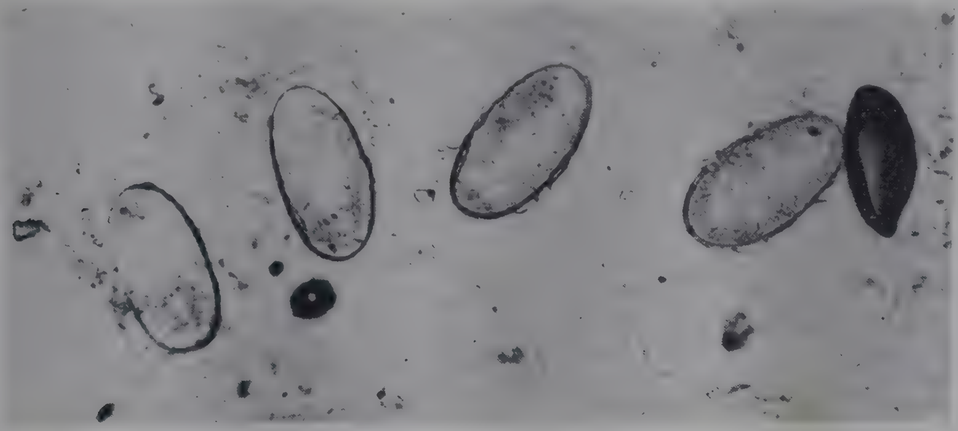

Fig. 580.—Diagnosis of infestation with the *Sarcoptes scabiei* var. ***hominis*** by the slice, scrape, and smear method. High power of Acarus eggs. (Official U. S. Navy photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in U. S. Nav. M. Bull. **46**: 834, 1946, by courtesy of the author and the publisher.)

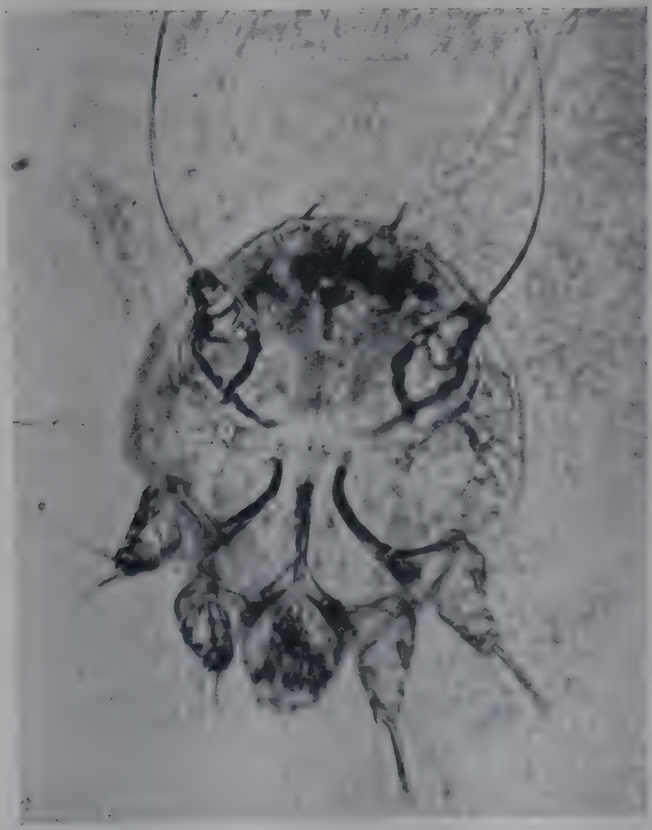

Fig. 581.—Diagnosis of infestation with the *Sarcoptes scabiei* var. ***hominis*** by the slice, scrape, and smear method. Hexapod Acarus larvae high power. (Official U. S. Navy Photograph. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in the U. S. Nav. M. Bull. **46**: 834, 1946, by courtesy of the author and the publisher.)

digital webs, on the hands, wrists, and axillae, use a sharp razor blade to shave off a very thin slice of the vesicle, burrow or cuniculus, or excoriated papulae. For lesions on the abdomen, genitalia, buttocks, and other parts less easily reached, scraping of the lesion with a sterile razor blade or Bard-Parker knife is best. Place the thin slice of epidermis, or the scrapings, on a glass slide, cover with a cover glass, and mount in 10 to 20 per cent potassium hydroxide solution, glycerin, water, or saline solution. Then examine the slide under low power, using the high power when needed.

Potassium hydroxide is used when one wishes to make a quick diagnosis as it rapidly breaks up the skin cells making it easy to see the Acarus or any of its parts or products. If saline solution or water is used it is possible at times to find a scabies organism near the surface of the skin examined and then evidence of vitality can be seen.

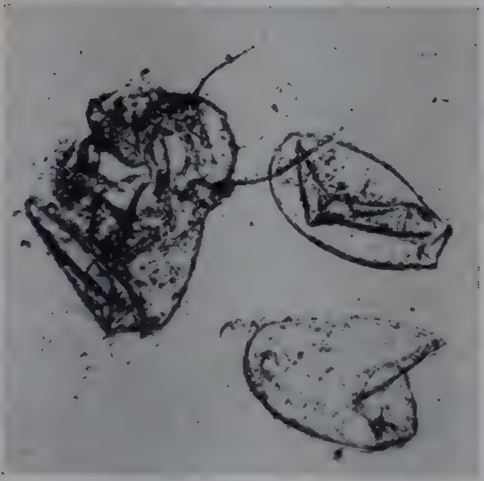

Fig. 582.

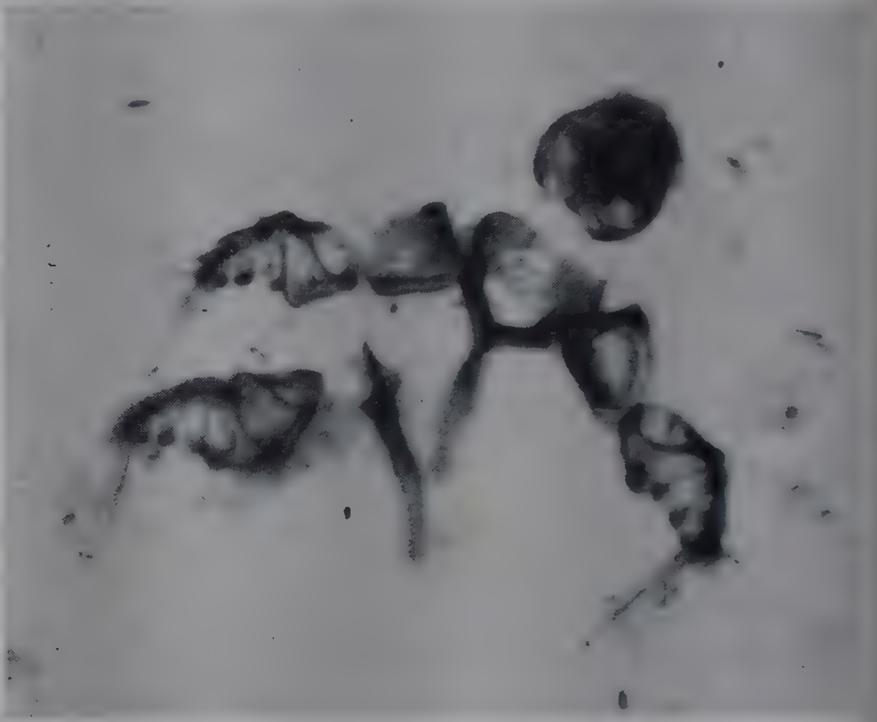

Fig. 583.

Fig. 582.—Diagnosis of infestation with the *Sarcoptes scabiei* var. *hominis* by the slice, scrape, and smear method. Empty Acarus eggs and larva emerging from egg. (Official Navy Plate. Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in the U. S. Nav. M. Bull. **46:** 834, 1946, by courtesy of the author and the publisher.)

Fig. 583.—*Sarcoptes scabiei.* Skeletal parts of the Acarus (all that is sometimes found in searching for the Acarus by this method). (Photomicrograph by Lt. Comdr. Eugene A. Hand, (MC) USNR, in U. S. Nav. M. Bull **46:** 834, 1946, by courtesy of the author and the publisher.)

Life Cycle (after Brumpt[4]).—The eggs hatch 7 days after being laid, releasing the hexapod larvae, which soon leave the burrow to live on the healthy normal skin where they enlarge and undergo several molts. The first molt occurs about the sixteenth day, giving rise to the octopod nymph which has 12 dorsal spines. These live in crusts on the surface of the skin and molt again about the twenty-first day. Another molt occurs about the twenty-eighth day, setting free pubescent males and females, which copulate. The fertilized females undergo further growth and development before becoming adults. About the sixth week a final molt occurs, and the female develops an oviduct for laying eggs. The female adult is pearly gray or pinkish and is

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[4] Brumpt, E.: Précis de parasitologie, ed. 5, Paris, 1936, Masson et Cie.

330 to 350 microns long and 250 to 350 microns wide. The female, fertilized, oviparous adult then burrows into the skin making its characteristic burrow, laying eggs as it goes. The eggs nearest the Acarus in the burrow are the ones most recently laid. The eggs measure 150 by 100 microns. The female egg-bearing adult inhabits the end of the burrow. The males at times live in the burrow but usually are found outside the burrow under the epidermis. The male is pinkish and measures 200 to 235 microns long and 145 to 190 microns wide. The male organism has a penis and sucker in place of the last pair of posterior legs. The male Acarus is a very elusive creature and is rarely seen.

Myiasis

(Parasitic Larvae of Flies)

Myiasis is a disease caused by larval flies in man and beast. There are two types: Myiasis interna and externa.

The larval flies are small, whitish-yellow to light brown, wormlike structures, usually consisting of 11 to 12 segments. The forward part is smaller than the posterior part. Each segment carries a crown of fine hooklets or hairs. The mouth shows two deeply stained sharp hooklets. On the posterior end are two dark spots which are the openings of the respiratory organs. The size, dependent upon the developmental stage, is from a few millimeters to 10 or 15 mm. Many varieties can be artificially grown in moist air to full development.

Myiasis Muscida

Myiasis muscida is caused by various species of Diptera, such as *Musca domestica, Fannia canicularis,* and *Calliphora vomitoria.* This is a common fly. It can lay its eggs upon inflamed or purulent wounds. The larvae wander into the cavities of the body and cause local disturbances with severe manifestations of disease. The cavities into which they have been known to wander are nose and sinuses, the ear, and the throat. They can even enter the stomach and from there go into the intestines and cause severe intestinal disturbances. In such cases, the larvae can be found in the stool. There have also been cases of larvae of *Piaphila casei* reported from the intestinal canal. These cases show severe disturbance, vomiting, pain in the abdomen, etc. The larvae are readily recognized in the stool.

Another variety is the *Cochliomyia,* or *Lucilia macellaria.* These flies are found in North and South America as well as in Asia. They enter the body of man, and have been reported in the bowels as well as in the vulva and penis.

Cordylobia Anthropophaga

Cordylobia anthropophaga are found in tropical West Africa. The larvae enter the skin of man and beast and cause considerable trouble. They cause painful pus collections in the arms, breast, head, and other places. The main symptom is itching of the skin, but severe pains are also noted. The lymph glands in the neighborhood of the larvae localization are swollen. The larvae reach their full development in eight days. According to Blacklock and Thompson, the wild rat is the principal host.

Auchmeromyia Luteola

Auchmeromyia luteola is found in West Africa, principally in the Congo. It is a blood sucker. It stays on the wall during the day and at night attacks the sleeping individual from whom it sucks blood. It appears light red from this blood content. The larvae measure about 10 mm.

Parasitic Larvae of Sarcophagidae

There is a variety of fly, the larvae of which enter ulcers and body cavities, particularly the intestinal canal and the eyes. In the eyes they produce a disease known as "ophthalmomyiasis."

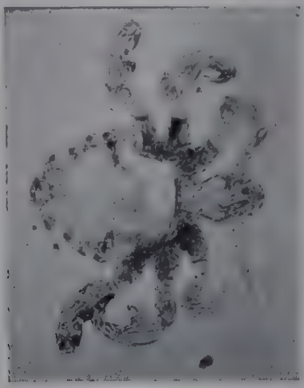

Fig. 584.—Crab louse of man.

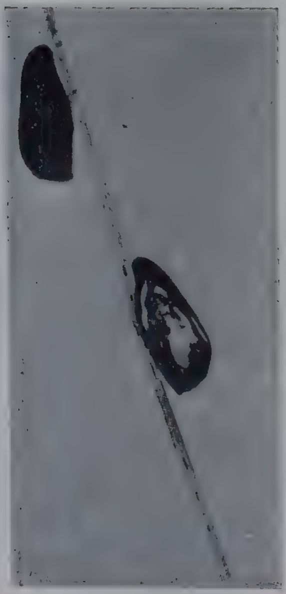

Fig. 585.

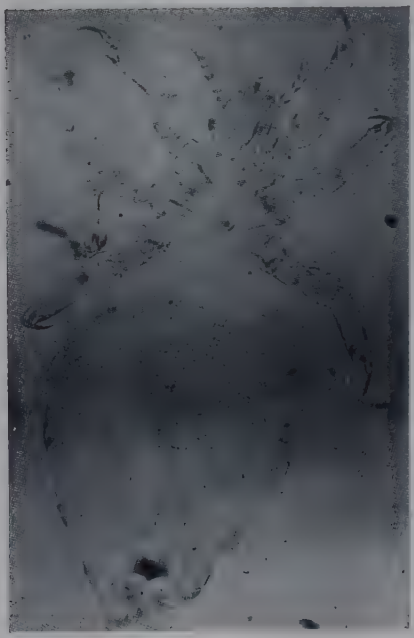

Fig. 586.

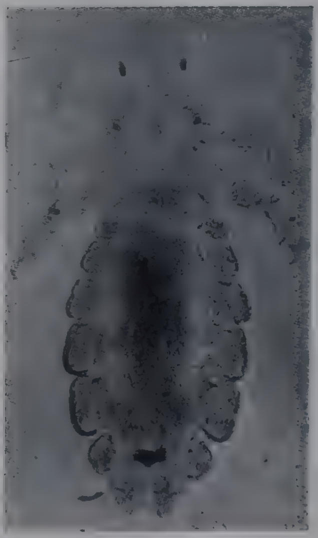

Fig. 587.

Fig. 585.—Eggs of crab louse, *Phthirius* (×30).
Fig. 586.—Body louse of man, *Pediculus vestimenti* (×30).
Fig. 587.—Head louse of man, *Pediculus capitis* (×30).

Parasitic Larvae of Oestridae (Myiasis Oestrida)

Oestridae usually create disturbance in their larval stage. The larvae are larger than those of the ordinary fly. They enter the skin and pass into the blood. There are several varieties of these flies.

One variety, *Dermatobia cyaniventris*, is seen in North and South America. The eggs are picked up often by the bodies of other flies and thus infect individuals. In South America the larvae are known as "berne" in Brazil, "ver moyoquil" in Mexico, "torsel" in Venezuela, and "nuche" in Colombia. These larvae produce swelling in the skin of man, which rapidly goes to abscess. The larvae then fall out and develop into flies. In man, these infections are found in the head and the back.

Other varieties of Oestridae are *Hypoderma bovis* and *H. lineata.* These are seen in Africa and North America.[1] In the skin there are elevated red lines which demonstrate the paths of the invading larvae.

Another variety is *Gastrophilus.* These larvae are usually in the stomach of the horse but sometimes enter the skin of man, producing a disease known as "larva migrans," seen in Russia and described by Wohasacif. These cases show slightly elevated red lines with little nodules. There is considerable itching of the skin.

Next variety of the Oestridae is the *Oestrus ovis,* or *Rhinoestrus nasales.* These larvae enter the nasal orifices. They are often seen in sheep, but seldom infect man. When they do, they enter the eyes, producing ophthalmomyiasis. Cases of this kind have been described in Asia Minor and Siberia.

The North American Chigger

The common North American chigger is the *Eutrombicula alfreddugesi* (Oudemans). Chiggers represent the larval form of certain mites. They are not only a pest, but a certain group of the genus Trombicula, found in the Southwest and Central Pacific, as well as other regions of the Far East, closely related to our own common chigger, is a transmitter of scrub-typhus. Williams[2] has given a description of a rapid collecting method for study of this larval form. We are indebted to him and to the Williams and Wilkins Company, Baltimore, Maryland, for permission to reproduce the cuts which appear with Dr. Williams' article. Two types of localities are typical haunts of the common "red-bug." They occur in greatest numbers under and near blackberry bushes, and also in large numbers near the base of pine trees, particularly at the base of those in groups of three or more. The explanation probably lies in the fact that ground-feeding birds and perhaps certain rodents are attracted to these spots to feed during the berry season. While there feeding, chiggers which have become engorged on their epidermal cells detach themselves and drop to the ground to continue development, as is their habit when they are fully engorged. Williams stated that it required only 25 females to produce the 10,000 chiggers which he collected in one summer. His method of collection was the so-called "white dish" method, made by placing a number of white saucers, usually from five to eight, where the chiggers were found in greatest numbers. The saucers were placed several feet apart. After a minute's exposure to the grass upon which they rested, several minute red specks could clearly be seen moving about rapidly over the white background. These chiggers were "sucked" into an aspirator, constructed from a glass vial or test tube, a two-hole stopper, two small pieces of glass tubing, and two short sections of rubber tubing, and brought back to the laboratory in a living state. Williams was able to collect 105 larval mites in 4 minutes 45 seconds.

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[1] See records of cases by Smith of Miami, Florida and Dover of the Bureau of Entomology, U. S. Department of Agriculture.

[2] Am. J. Trop. Med. 26: 243, 1946.

While chiggers do not burrow into the flesh, they attach themselves to their host in a manner similar to ticks. They evidently form a tubelike structure which extends more or less deeply into the skin from the mouth parts of the mite larvae. This tube in mammals, including man, is comparatively short, without branches, and lined by a distinct layer of cells. In parasitized mosquitoes this tube is fairly long and without branches, but in spiders, there are

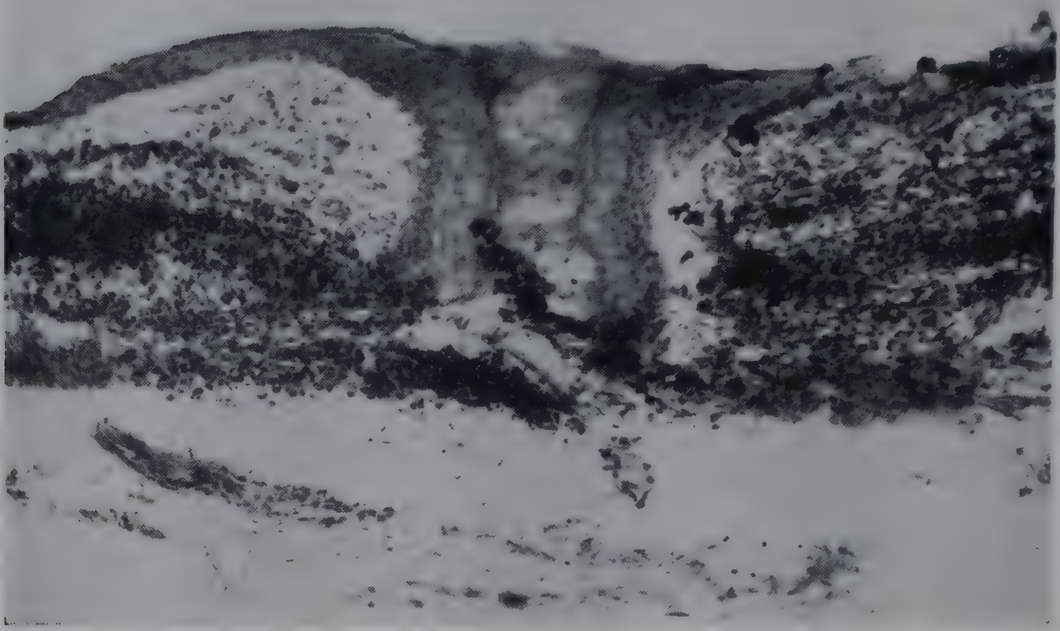

Fig. 588.—Histologic preparation of a chigger bite in a rabbit ear. Section of a bite showing the tube lined with stratum germinativum cells and containing partially digested exudate of tissue fluids and cells. (Photomicrograph by Roger W. Williams, from Am. J. Trop. Med. **26**: 2, March, 1946, by courtesy of the author and the publisher.)

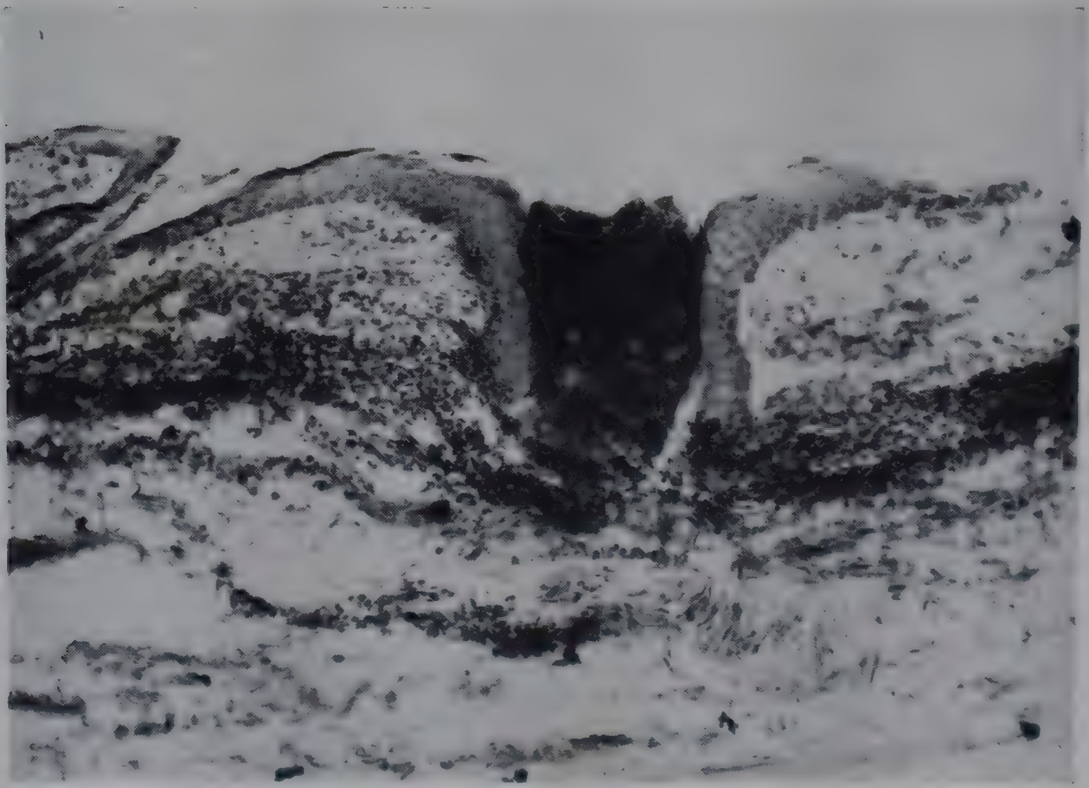

Fig. 589.—Histologic preparation of a chigger bite in a rabbit ear. An infiltration of cells and fluid being "sucked" through the inner end of the tube. (Photomicrograph by Roger W. Williams from Am. J. Trop. Med. **26**: 2, March, 1946, by courtesy of the author and the publisher.)

ramifications. Histologic preparations of chigger bites on rabbit ears show (Fig. 588) that the epidermis is completely penetrated. A tube lined with stratum germinativum cells is formed which extends to the derma and subcutis. This tube appears to represent a reaction of the host to the secretion of the parasite; its inner layer of cells is necrotic and gives evidence of digestion. There is some degree of round cell infiltration in the area of the tubelike structure. After removal of the chigger the tube contains a partially digested exudate of tissue fluids and cells. The chief source of food is derived from an infiltration of cells and fluids which are ''sucked'' through the inner end of the tube formed by the protective wall of stratum germinativum (Fig. 589). The protective wall grows and eventually closes off the opening of the inner end of the tube which it forms (Fig. 590). As the liquefied center or homogeneous zone dries and contracts, it may drop off or be pulled out as a whole from the bite (Fig. 590).

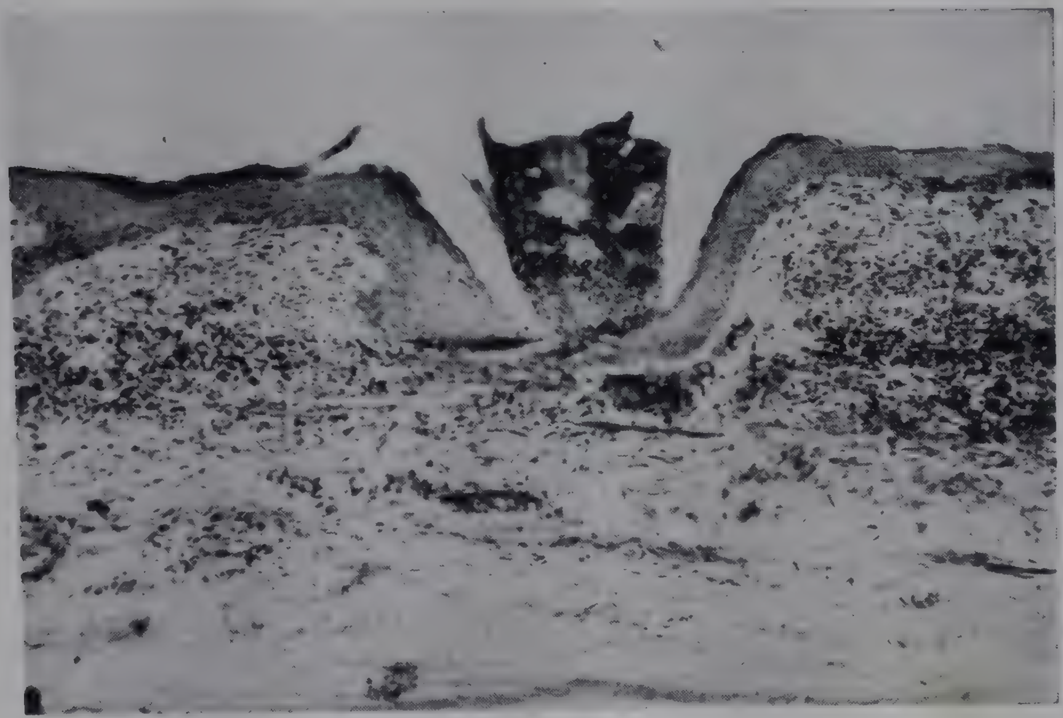

Fig. 590.—Histologic preparation of a chigger bite in a rabbit ear. The protective wall closes off the opening at the inner end of the tube which it forms and when dried the homogeneous zone may drop or be pulled out as a whole. (Photomicrograph by Roger W. Williams, from Am. J. Trop. Med. 26: 2, March, 1946, by courtesy of the author and the publisher.)

Rapidity of movement of these larval parasites is quite remarkable. At a temperature of 30.3° C. these larval mites can run, on the average, a distance of slightly better than one foot per minute.

Rarely do chiggers feed on blood and never is it their chief source of food. An extraoral digestion of epidermal cells is practiced by this parasite. Moreover, chiggers overwinter in the larval stage deep in the ears and on the eyelids of rabbits, squirrels, and possibly other animals. They are negatively geotropic and possess amazing speed for their size. Furthermore, they are frequently preyed upon by members of the mite family Bedellidae.

Latrodectus Mactans—The Black Widow Spider

The black widow spider has a widespread distribution throughout the southern half of the United States. These spiders are found in the angles

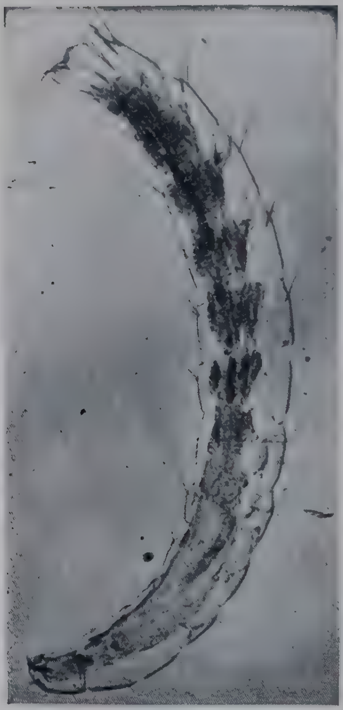

Fig. 591.

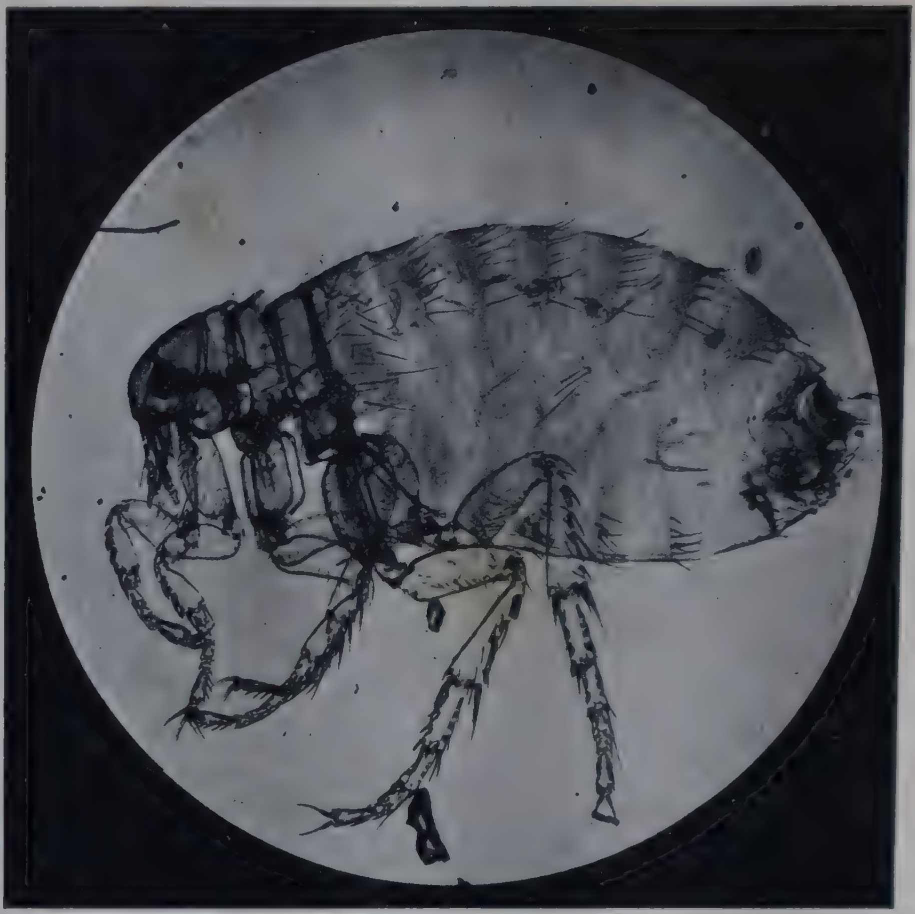

Fig. 592.

Fig. 591.—Larva of flea, *Pulex* (×30).
Fig. 592.—Human flea, *Pulex* (×30).

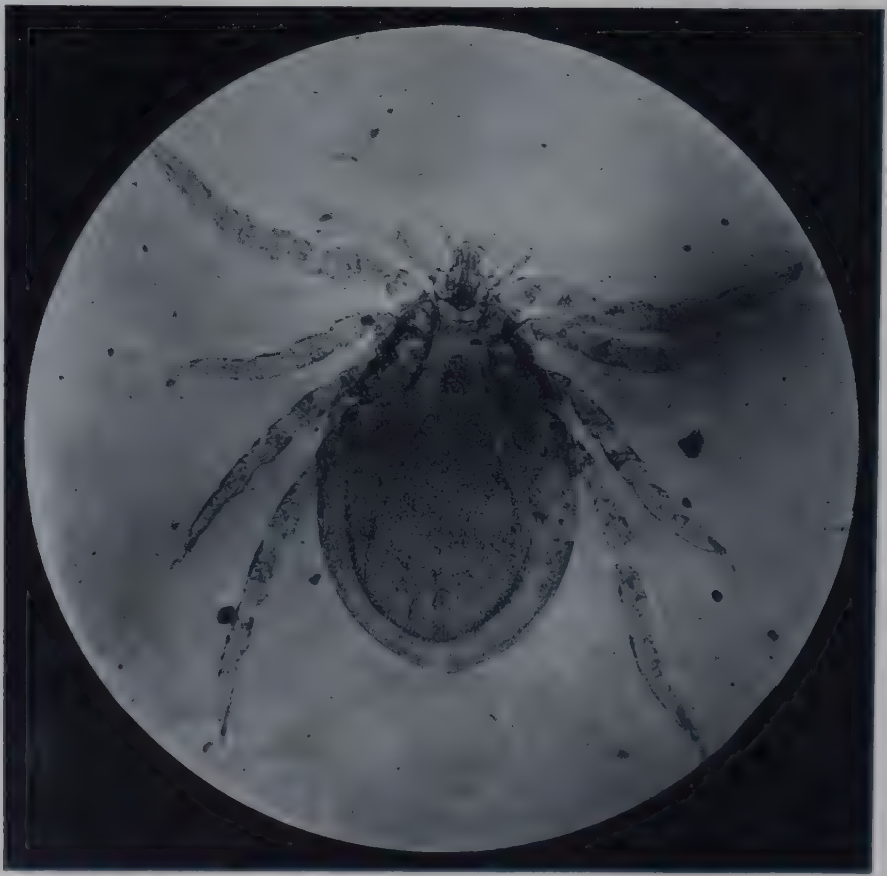

Fig. 593.—Sheep tick, *Ixodes* (×30).

between the roots of trees, beneath rocks, around old stumps, in holes in the ground, in outhouses, and outbuildings. They have been found in the greatest numbers in the vicinity of human habitations. Occasionally they have been found in dark corners and in enclosed parts of dwellings. They prefer dry, dimly lit places.

The black widow is a nocturnal spider, practically all bites reported having occurred in the evenings or at night, in outdoor toilets where the spider has woven its irregular web beneath the seat, or in dry, dark corners of garages or woodsheds.

The web is so distinctive that once it has been seen it may be recognized again at a glance. It is composed of irregularly arranged, coarse threads which crisscross at various angles and in different planes. It is always found built in relation to a crevice, corner, etc., in which the spider frequently, by means of a poorly formed tunnel of silk, hurriedly retreats when disturbed. The web is relatively very strong and appears to entangle insects many times the size and weight of the spider. On many occasions, there have been observed large wood and water beetles in the web; in one case, a young mouse became entangled and was swathed and suspended in the web by these spiders. The web spun by the male is always smaller and weaker than that of the female.

Black widow spiders are capable of going without food for over a month with no recognizable harmful effects. Their fluid requirements are supplied by the body fluids of their victims. While awaiting their prey, they usually are found hanging in their webs with their ventral surface uppermost. They are cannibalistic, feeding upon each other whenever the opportunity presents itself. The common nickname, ‘‘black widow,’’ given to the female of this species, arose from her habit of capturing and feeding on the much smaller male.

The spiders are equipped with a poison apparatus for purposes of killing their prey. The poison apparatus of *Latrodectus mactans* consists of two poison sacs, each with an attached duct leading into the heavily chitinized chelicerae or horny fangs through which the poison is expelled by biting. Since the mature male does not attack prey, the poison apparatus appears to become inactive and remains small. In the female spider, on the other hand, this apparatus increases in size and strength with maturity.

The fatal dose of the poison injected subcutaneously or intravenously is extremely small. Cats are killed by quantities equal to 0.2 to 0.35 mg. per kilogram of body weight.

Effect of the Bite on Man.—The bite, itself, similar to a pinprick, is not always felt, and often there is but little evidence of a lesion. However, a slight local swelling and two tiny red spots may occur, and local redness is usually in evidence at the point of attack.

Pain in the region of the bite is felt almost immediately, and increases in intensity, reaching its maximum in 1 to 3 hours, and generally continuing for 12 to 48 hours, gradually subsiding. Rigidity and spasm of most of the larger muscle groups of the body, particularly of the abdomen, are most notable. The abdominal muscles become ‘‘boardlike,’’ but local tenderness as in appendicitis is almost always absent. There is a slight rise in body temperature, increased blood pressure, and also, as Bogen reports, a definite leukocytosis, and usually an increase in the pressure of the spinal fluid. Profuse perspiration is evident and there is often a tendency to nausea.

In man, the bite is followed, with dramatic suddenness, by a characteristic and alarmingly severe systemic reaction.

There is little doubt that the bite of the black widow spider is much more serious than that of other arachnids, and depends in effects on both the condition of the spider and the person bitten. The extract from a single spider is sufficient to kill 1,000 cats.

Diagnosis.—Diagnosis of araneism due to the bite of *Latrodectus mactans* depends upon:

(1) History of exposure to, or actual experience of, the bite of the black widow spider. There is usually little or no visible lesion at the site of the bite.

(2) Pain, beginning usually in the region of the bite but not always, and spreading until most of the larger groups of muscles of the body are involved. Except in pregnant multiparous women, the pain is usually felt most intensely in the abdominal muscles. The pain increases, reaching its maximum about an hour after the bite. It is then evidently excruciating and agonizing, usually continuous, but occasionally cramplike or intermittent, and manifested by writhing, doubling up, and screaming or moaning.

(3) Rigidity and muscle spasm occur, most notably in the muscles of the abdominal wall, which become boardlike and hard, as in general peritonitis. It should be noted, however, that this rigidity is not confined to the abdomen, but that the intercostal muscles may contract, causing backache or stiffness in the back. Spasm in muscles of the extremities may be marked, and the legs may be drawn up on the abdomen. True local tenderness, however, is usually entirely absent, or disproportionately slight compared with the intensity of the muscle spasm.

(4) Increased blood pressure, usually 20 to 40 mm. above the normal for that patient, hyperactive reflexes, especially the knee jerk, and an increase in the pressure of the spinal fluid are usually present.

(5) A mild rise in body temperature, with occasionally, but not uniformly, a slow pulse, and a definite leukocytosis, with an increased percentage of neutrophiles, is found.

(6) Profuse perspiration, restlessness, and anxiety, tossing about in agony are usually symptoms.

(7) Nausea and vomiting, localized edema, twitching or spasms of the muscles of the extremities, and urinary retention are occasionally noted.

If these considerations are borne in mind, it is not usually difficult to distinguish a case of araneism from one of an acute surgical abdominal condition, renal or biliary colic, tabetic crises, food poisoning, pneumonia or pleurisy, tetanus, or any of the other conditions that may be suggested by the clinical symptoms.

Diagnosis and Treatment of Scorpionism and Araneism[1]

Both in tropical and in subtropical countries the laboratory technician may be called upon to help clinicians either to establish a diagnosis of some rather puzzling conditions in patients or to treat them. Often patients step into physicians' offices quite hurriedly and complain of "terrible pains," or tell stories of having been bitten or stung by a "small, nonflying, secretive animal."

In such cases it is advisable to maintain a critical attitude of mind, and to think first of the possibilities of scorpion sting or spider bite.

Scorpionism.—Scorpions sting as a rule in warm weather, at night, or in dark places, such as closets, barns, storage rooms, and cellars, since these little animals like to seek shelter in boots and shoes, piles of hay or wood, among bottles, etc.

Symptoms.—In most cases the principal symptoms are:

a. **Local**: A reddish white-centered wheal surrounding the **single puncture** (caused by the sting at the tip of the scorpion's tail) and spreading into an edematous area of serous or bloody aspect; pain, very intense and sometimes excruciating, with a tendency to extend along the affected limb to the chest;

b. **General**: Slow pulse as a central nervous reflex to the pain; repeated sneezing, watery nasal and bronchial discharges; profuse lacrimation accompanied by mydriasis, nausea, vomiting and diarrhea, profuse perspiration and cold sensation, trembling and dizziness, as an effect of the venom on the sympathetic nervous system.

In some cases, particularly in nervous children or whenever the amount of the venom inoculated by the sting is large enough, there may appear a final or depression stage, represented by a marked exaggeration of the reflexes, particularly the patellar; clonic contractions, sensation of throat constriction, hiccup and convulsions, showing the direct and late action of the venom neurolysin on the central nervous system; tachycardia and low blood pressure, dyspnea, hypothermia, coma and, eventually, death. In other cases, such as those caused by the sting of the African *Buthus occitanus,* and other species, and the Brazilian *Tityus serrulatus,* these symptoms are aggravated by hemorrhages through the mucosa of the gastrointestinal duct (hematemesis and melena) or through the kidneys (hematuria), showing the destructive action of the venom proteo-hemolysin of these species on both the endothelial cells of the capillaries and the red cells of the blood.

Treatment is based on the application of a specific antivenin, either hypodermically or intravenously. Since scorpion venoms, like snake venoms, vary from species to species, it is advisable to use only an antivenin or antiserum that has been prepared from the venom of local species. The usual dose is 10 to 30 c.c., children requiring a larger amount of antivenin than adults, in view of their lower weight, to neutralize the relatively greater concentration of the venom in their systems.

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[1] Contribution by Afranio do Amaral, B.Sc. & Lit, M.D. (Bahía) D. Hyg. & Trop. Med. (Harvard), Founder and Director, Antivenin Institute of America (Pa., U. S. A.), Co-Founder and Professor, Escola Paulista de Medicina (São Paulo, Brazil) Lecturer, Harvard University School of Public Health, Reorganizer and former President, São Paulo Red Cross, Member, International Commission on Zoological Nomenclature (London), Director, Instituto Butantán, São Paulo, Brazil.

Whenever a specific antivenin is not available, it is recommended to take into consideration the neurotropic and sympathico-mimetic effects of scorpion venoms in order to attempt to counteract them. Therefore, at the invasion period of the poisoning, the patient should receive, about the sting puncture, an ampule of 1 or 2 c.c. of magnesium sulphate solution with Novocain (magnesium sulphate 25%, distilled water 74.5%, Novocain 0.50%) so as to relieve pain. In the excitation period, in order to neutralize the adrenergic effects of venom, injections of acetyl-choline, by acting on the postganglionic fibers of the para-sympathetic system, usually bring the patient some comfort. In the depression period, the administration of stimulants and cardiotonics is advisable particularly when supplemented by liberal amounts of normal saline *given warm and slowly* by the venous route and glucose solution or Ringer-sodium acetate solution, also intravenously, given warm and slowly.

In mild cases it suffices to give the patient an intravenous injection of 10 c.c. of a 10 per cent calcium gluconate solution in order to relieve pain. In rare instances, whenever there is some reason to believe that an acute pancreatitis is complicating the clinical feature, 5 to 10 units of insulin are indicated to complete the treatment.

In view of the sympathicomimetic action of scorpion venoms, administration of any strong heart stimulant is contraindicated during the excitation period of the envenomation. Applications of tourniquet, bleeding, and suction are of no avail whatsoever. Use of potassium permanganate does not help the patient in any way.

Araneism.—Spider bites as a rule may occur at any time, both in tropical or subtropical countries and in temperate regions. Spiders, being secretive, unsociable animals which seek shelter under dead trees, dry leaves, and piles of hay, sometimes invade toilets, closets, kitchens, cellars, stables, and barns to catch insects, mice, and other small forms on which they feed. In some places they spin their webs under the cover of outdoor toilets where they can frequently capture flies. In this case they may bite humans at or around the genitalia; otherwise, their bites usually take place on the feet or hands and, eventually, under the arm or elsewhere.

Leaving aside the Mygales or crab-spiders (*Mygalomorphae*), which in general are not very dangerous, the true spiders (*Aranomorphae*) are provided with a pair of fangs moving horizontally (so as to converge at the moment of the bite), each fang corresponding to an elongated poison gland.

For this reason the bite caused by any spider usually leaves on the patient's skin a double marking or **two punctures**, the symptoms that follow the introduction of the venom varying from species to species according to the chemical composition of this secretion.

From a physiopathologic standpoint, spider venoms may be grouped into the following categories: (a) neurotropic; (b) hemolytic and icterogenic; (c) dermotropic or necrotic.

Virtually all spider venoms are neurotropic, some being more so than others. The species whose venoms are most typically neurotropic are, for instance, *Ctenus nigriventer,* the "armadeira" of Brazil and *Latrodectus mactans,* the "black widow spider" of the United States, also called "casampulga" in Mexico and Central America, "lucacha" in Peru, "guina" in Chile, and "araña-deliño" in Argentina.

The venom of *Lycosa raptoria* of Brazil, Uruguay, and Argentina, is characteristically dermotropic or necrotic.

The venom of *Epeira diadema,* of the West Indies, is both neurotropic and hemolytic, although devoid of any icterogenicity. That of *Araneus audax,* of the meridional section of South America is necrotic, hemolytic, and icterogenic, while that of *Glyptocranium gasteracanthoides,* the "podadora" of Peru, is hemolytic, necrotic, and slightly neurotoxic.

From the above it is clear that besides the double puncture, which is their only common feature, clinical symptoms of araneism vary quite widely from species to species

Latrodectus Mactans.—Local red wheal, pain, swelling and numbness, followed by fever, depression, shivering, restlessness, convulsions, spasms and rigidity of the muscles of the abdominal wall and other regions; vomiting, hypothermia; sexual excitation and anuria and death in severe cases.

L. Hasselti.—Some of the *L. mactans* signs of poisoning followed by internal hemorrhages and icterus.

Ctenus Nigriventer.—Acute pain, muscular spasms, chills, cold perspiration, salivation and lacrimation, followed by dysuria, tachycardia and arterial hypotension, sometimes terminating in death especially among children.

Lycosa Raptoria.—Extensive edema with phlyctenules and destruction of the derma at the site of the bite, followed by sloughing of the affected tissue and an irregular scar.

Epeira Diadema.—Pain followed by bloody swelling at the site of the bite and hemorrhages through mucous membranes; hyperthermia and nervous depression.

Glyptocranium Gasteracanthoides.—Red wheal and papula surrounding the punctures, spreading phlegmonous edema with phlyctenules, sloughing of the derma and necrosis of the affected tissue followed by deep scar; tachycardia, dyspnea and depression; hematuria sometimes complicated by anuria.

Treatment of such a complex symptomatic picture must of necessity be directed by a correct diagnosis and by the evolution of each case as well.

Specific therapy by means of antivenin must be applied whenever it is possible or in severe cases especially in children. Unfortunately only very few laboratories have considered the necessity of producing antivenins specific for local spiders.

Should a specific antivenin be available it must be given, either locally (intradermal injection) in case of a dermotropic poisoning, or hypodermically in other cases, and intravenously in children or in any severe case, a dose of 5 to 20 c.c. usually being sufficient.

Nonspecific therapy consists of the following: (a) against pain and local swelling: warm dressing with concentrated solution of magnesium sulphate;

sometimes an intravenous injection of 5 or 10 c.c. of a 10 per cent calcium gluconate solution causes a lasting relief; (b) against depression and arterial hypotension: cardiotonics and general stimulants such as caffein and epinephrin; (c) against hemolysis (and icterus): liver extract and blood transfusions; (d) against spasms (and cramps): an injection of atropine to block the parasympathetic, followed by neostigmine to cause muscular relaxation.

CHAPTER XIII

SEROLOGY

This chapter will take up the subject of the diagnosis of disease by those serologic methods known as complement fixation and precipitation technics. Complement fixation technic really had its beginning with the discovery of Pfeiffer in 1894 of what has been termed the "Pfeiffer phenomenon" or bacteriolysis. Pfeiffer showed that, with the development of immunity by an animal to bacteria, its body fluids develop the property of dissolving these bacteria. He immunized guinea pigs to cholera organisms. After immunizing these animals, he injected the same cholera vibrios into their peritoneal cavities and found, when he punctured the abdominal cavity with sterile glass capillary tubes and withdrew peritoneal fluid, that this fluid dissolved the cholera vibrios. Nonimmunized pigs did not show this phenomenon. This was an example of bacteriolysis. Metchnikoff further proved that this same phenomenon could be detected in the test tube; i.e., by mixing cholera vibrios in a test tube containing the peritoneal fluid of an immunized guinea pig, the same bacteriolysis could be observed. Bordet showed that upon heating the cholera-immunized serum to 56° C., adding fresh unheated blood serum, plus cholera organisms, bacteriolysis occurred; upon heating the immune serum, not adding blood or serum, and adding cholera organisms, there followed no bacteriolysis. Thus he demonstrated that there were two substances concerned with the bacteriolytic test; viz., a specific thermostable antibody which could withstand heating to 56° C., and another which could not withstand such heating—and this latter substance which is thermolabile could be replaced by fresh normal blood or blood serum. He first showed that the thermolabile substance is present at all times in normal blood and is not a product of the immunization injections, but the substance which is thermostable is a product of immunization injections and is not normally found in blood or blood serum. This phenomenon of bacteriolysis was found similar to that of hemolysis which was first noted by Bordet and Gengou[1] in their classic article, entitled, "Sur l'éxistence de substances sensibilatrices dans la plupart des sérums antimicrobiens." In this article they showed that hemolysis could be developed by the interaction between a substance, such as sheep cells, and a corresponding "substance sensibilatrice" produced by injecting a rabbit with repeated quantities of sheep cells in the presence of a third substance called *complement*. In other words, an antibody formed by the organism in response to the injection of the sheep cells, the "substance sensibilatrice" now called generally hemolysin (or amboceptor), would hemolyze the sheep cells in the presence of a sufficient amount of *complement*—an unknown species-specific substance found in various quantities in practically all animals. It was further shown that the heating of the serum containing hemolysin, at 56° C.

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[1] Ann. Inst. Pasteur 15: 289, 1901.

for one-half hour destroyed the complement substance but did not affect the antiserum or hemolysin in any way. It was upon this so-called hemolytic system, which was only mildly interesting to research workers for a number of years, that Wassermann, Neisser, and Bruck[2] based the famous test, now named after the first one of these investigators, which is commonly considered the classic serologic test for the diagnosis of syphilis. Wassermann, Neisser, and Bruck believed that in the blood of all syphilitic patients there was an antigenic substance, or antibody, which in the presence of a specific extract of the *Treponema pallidum* was sufficient to so bind or fix complement that the later addition of the hemolysin and the homologous cell (sheep cell) was followed by no hemolysis or lack of hemolysis; furthermore, that if the blood serum came from a patient who was not syphilitic, the antigenic extract supposedly derived from a suspension of *Treponema pallidum* would not bind complement, which would be free later to hemolyze the specific hemolysin and cellular mixture (sheep cells). This was a very valuable application of the well-known Bordet-Gengou phenomenon and has served exceedingly well throughout the years since 1906 to assist us in the diagnosis of syphilis. Strange to say, the theoretical assumption by Wassermann and his co-workers that the blood serum of the syphilitic contained the syphilitic antibody and was bound by a supposed extract of the *Treponema pallidum* has been found to be in no sense theoretically correct. The original extract of the *Treponema pallidum* introduced into the Wassermann system by the originators of the test was a tissue extraction of liver substance derived from congenital (stillborn) syphilitics. It was found soon after the announcement and use of their test that equally specific results could be obtained with the use of liver tissue extract not derived from syphilitic tissue at all. The use of tissue extracts in alcohol or ether derived from normal organs, such as human heart, ox heart, ox kidney, etc., yielded excellent antigenic extracts for the performance of the Wassermann test. The diagnostic value of the Wassermann test cannot be attacked, yet the theory which led to its discovery has been shown to be completely erroneous. The Wassermann reaction and its modifications are quite specific diagnostic tests, yet the substance in the syphilitic serum which is responsible for the fixation of complement, or for precipitation flocculation, is not definitely known. The term **reagin,** or the substance which reacts, was applied by Citron[3] to the active factor of syphilitic serum. It is a very useful term to so designate the substance which is found in syphilitic blood serum and spinal fluid and which is responsible for the specific reaction. Soon after the discovery of methods of cultivating *Treponema pallidum* by Noguchi, an attempt to make a specific antigenic extract from cultures of the supposedly growing organism failed to produce a laboratory reagent which in any way compared in efficiency with the various types of alcoholic extracts of mammalian tissues.

It is sufficient to say at this point, therefore, that the serologic methods which we employ in the diagnosis of syphilis are quite useful and reliable although the theory which truly explains the specificity of these reactions still remains to be promulgated.

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[2] Deutsch. med. Wchnschr. 32: 19, 745, 1906.
[3] Berl. klin. Wchnschr. 44: 1370, 1907.

The methods of making a serologic diagnosis of syphilis consist in the main of two types of tests, the complement fixation type and the precipitation type. There are many modifications of the original Wassermann and many types of precipitation tests. There are adherents of both the complement fixation and the precipitation methods. There has been much dispute regarding the value of each type of test and also as to priority of discovery. It seems to the author that the most valuable opinion on the value of the various types depends largely upon the experience of the worker. It has been quite interesting since my early days in Wassermann's laboratory in 1908 to view the various developments of the test, the improvements in the technic, the eradications of faults, and development of precipitation methods. The modifications of the Wassermann test now in use are quite specific and the various types of precipitation tests are equally specific. Those methods will be detailed which, in our hands and in the hands of other standard workers, have proved most efficient and reliable.

EXPLANATION OF TERMS USED

1. Blood Serum

Blood serum is the fluid part of the blood which separates from clotted blood. It is usually straw colored, and is free of cells. It is this part of the blood which is used in serologic diagnosis. Certain facts about this material must be taken into account in a consideration of complement fixation tests. Patient's serum (unheated) contains variable amounts of complement and hemolysin. Normally, it shows naturally no syphilitic substance. It may or may not show syphilitic substance during an infection with syphilis, or rather, the amount varies under various conditions and under the influence of treatment. The heating of blood serum to 56° C. destroys complement, but does not destroy hemolysin. It has also been proved that this heating, which is called ''inactivation'' in the technic, has a destructive effect on syphilitic substance in certain individuals. This is due to the fact that the syphilitic substance is composed of two kinds of material, one ''thermolabile'' and one ''thermostable.'' The relative proportions of these two different kinds of syphilitic substances are variable, so that in order to retain the maximum amount of both substances, the serum should preferably be examined for complement fixation in an unheated state. Since the Wassermann technic entails the use of heated serum, necessarily, at times, the process of inactivation, or heating, will destroy the very substance the presence of which we are trying to prove. In technic such as Hecht-Gradwohl, where unheated serum is used, the entire amount of unaltered syphilitic substance is determined.

2. Physiologic Saline

Physiologic saline is an 0.85 per cent solution of sodium chloride in water.

3. Inactivation

Inactivation is the heating of blood serum in a water-bath at 56° C. for one-half hour for the purpose of destroying complement.

4. Complement

Complement is a thermolabile, nonspecific, enzyme-like substance contained in the blood serum of all animals. The quantity of complement varies from time to time in the bodies of animals, and may disappear altogether after removal from the animal tissues. It takes part in the reaction between antibodies and antigen, and between hemolysin and its specific cells. It is present in particularly large amount in the blood of guinea pigs.

5. Antigen

The term "antigen" is an immunologic expression which actually means any substance "foreign" to the animal into which it is injected and which stimulates antibody formation by the injected animal. Originally, Wassermann advised the use of an antigen made from the liver of a congenital syphilitic child; that is, from the stillborn children of syphilitic parentage. In the Wassermann technic, the term "antigen" has come to mean an extract supposedly of substances referable to the *Treponema pallidum,* which are specific in their reaction with the antibody or immune body found in syphilitic blood serum; i.e., antigen and syphilitic immune body of homologous materials are mutually attracted in the presence of complement and are bound by complement, thus producing the phenomenon of "complement fixation." As a matter of fact, in the Wassermann reaction, the antigen used is not made from a syphilitic spirochete but is made from lipoidal material. It is for this reason that the Wassermann test is not really an antigen-antibody reaction but rather a very important test which is dependent upon factors other than the binding of complement by true antigen and true antibody. The term "antigen" in this sense is, therefore, a misnomer, but has been retained with this understanding.

The antigens used today in the Wassermann test are made by alcoholic extraction of normal organs, from alcoholic extraction of normal organs reinforced with cholesterin, from acetone-insoluble lipoids, from cholesterinized and lecithinized alcoholic extracts of heart muscle, and from cardiolipin.

A true antigen as prepared by Noguchi, using pure cultures of *Treponema pallidum,* is not as serviceable as any of the foregoing. The *T. pallidum* immobilization test is described on page 2085.

Antigens for complement fixation tests other than tests for syphilis are extracts of the organisms which cause the disease. For example, the complement fixation test for gonorrhea utilizes an extract of gonococci as an antigen.

In making serologic tests, new lots of antigen should be checked against old lots on two different days before using the new antigen. If comparable results are not obtained, the new antigen should not be used.

The Chemical Nature of Syphilis Antigen—Cardiolipin.—

Fischer and Velasco,[1] School of Anatomy and Physiology, University of Chile, Santiago, reported their results on various methods of purifying the syphilis antigen. In 1942 Pangborn[2] described a method of purifying the syphilis antigen of alcoholic beef heart extract by precipitating the phosphatides with cadmium chloride, suspending the precipitate in petroleum ether, and eliminating the lecithin-cadmium chloride complex by extractions with 80 per cent alcohol. The remaining solution contained cephalin-cadmium chloride complex and antigen. After eliminating the cadmium by ammonia, and cephalin by precipitations with alcohol, the antigen was further purified until a substance which Pangborn claimed to be the pure syphilis antigen was obtained. According to this author, this substance, called **cardiolipin,** is a nitrogen-free, sugar-containing phospholipid, with 4.11 per cent phosphorus.

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[1] Science 103: 455, 1946.

[2] J. Biol. Chem. 143: 247, 1942.

In further publications,[3, 4] Pangborn described another method of purifying the syphilis antigen, based on the precipitation of the cephalin fraction together with the antigen by barium chloride, and eliminating cephalin by alcohol. After purification a substance was obtained with the characteristics of syphilis antigen. This second ''cardiolipin'' contained no sugar but approximately the same amount of phosphorus as the ''cardiolipin'' of the first publication.

Between 1931 and 1936,[2] one of the authors and his co-workers elaborated several methods of purifying the syphilis antigen. Having applied two of these methods successively, a purified antigen preparation was obtained from the phosphatid fraction of alcoholic beef heart extract. The process of purification consisted of adsorbing the antigen on specially prepared aluminum hydroxide and eluting it in benzene. The antigen was then further purified by extracting it with petroleum ether from an acidulated 80 per cent alcohol solution. From 207 grams of phosphatides 1 gram of a purified antigenic preparation was obtained, with only 0.01 per cent phosphorus, 0.25 per cent reducing substances (liberated by acid hydrolysis), and 40 per cent fatty acids (liberated by alkaline hydrolysis).[5]

It was shown in 1936[6] that by extracting an 80 per cent alcoholic solution with petroleum ether, only a part of the antigen could be obtained in the petroleum ether solution, another part still remaining in the hydroalcoholic phase. Only after acidulating this phase with hydrochloric acid did the antigen disappear completely from it, entering into the petroleum ether phase. It may be assumed, therefore, that the antigen occurs in two different forms in the alcoholic organ extract; i.e., acid and salt.

In a later experiment, these authors worked on the phosphatid fraction of 10 beef hearts, obtained by extraction with alcohol and precipitation with acetone. The antigen was adsorbed on aluminum hydroxide and eluted in benzene. The substance obtained in this way was dissolved in 80 per cent alcohol and extracted at first without, and then with, acidulation.

They obtained in this way two fractions, which they called ''petroleum ether fraction before acidulation'' (P.E.N.) and ''petroleum ether fraction after acidulation'' (P.E.A.). Both fractions reacted with strongly positive sera. P.E.N. weighed 39 mg., and no phosphorus could be detected in it. P.E.A. amounted to 22 mg. with 0.1 per cent phosphorus. Both fractions were yellow oils which hardened rapidly, forming a transparent, soft film.

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[3] J. Biol. Chem. 153: 343, 1944.

[4] J. Biol. Chem. 157: 691, 1945.

[5] Fischer, E., Fischer-Dallmann, R., and Boné, R.: J. Immunol. 51: 117, 1945.

[6] Fischer, E., Steinert, J., and Fischer-Dallmann, R.: Ztschr. f. Immunitätsforsch. u. Exper. Therap. 89: 133, 1936.

They assumed that the small quantities of phosphorus found in their preparations correspond to impurities, as was the case with the reducing substances in the former experiment.

6. Complement Fixation ("Wassermann Reaction")

The laboratory diagnosis of syphilis comprises mainly two methods: first, the dark-field demonstration of the *Treponema pallidum,* for the diagnosis of primary sores; and, second, the serologic or serum diagnosis of syphilis. The dark-field examination has been described on pages 11 ff. The serum diagnosis of syphilis may be divided into two parts: first, the complement fixation, or Wassermann, reaction; and second, the flocculation, or precipitation, test.

Regarding complement fixation methods, of historical interest is Bordet's discovery of the hemolysins in 1898 and his demonstration of the roles of the antibody and alexin in the process. Bordet's discoveries were confirmed by Ehrlich and Morgenroth. Later, Bordet and Gengou brought out the "Bordet-Gengou Phenomenon."* Bordet showed that the complement in a normal serum would unite with either a bacteriolytic or a hemolytic amboceptor, and that, by furnishing sufficient of either amboceptor, all the complement may be "fixed." Prior to this Bordet had shown that the addition of a small amount of normal serum to an immune hemolysin would result in lysis of the homologous corpuscles, and that the process could not take place without the alexin. In their researches Bordet and Gengou gave the results attained with three different antigens and their respective antisera—pest, typhoid, and *Proteus vulgaris.* Gengou[1] later showed that not only cellular antigens, such as bacteria and red cells, are capable of stimulating the production of amboceptors, but that the proteins in solution, such as serum and milk, may produce complement-binding amboceptors in addition to precipitins. Widal and Lesourd[2] applied the Bordet-Gengou reaction to the diagnosis of typhoid fever, using an emulsion of typhoid bacilli and the serum of a typhoid fever patient; they found that a positive reaction occurred more frequently and earlier than the agglutination test. Wassermann, Neisser, and Bruck[3] applied the complement fixation method to the study of syphilitic infections and published their epoch-making report of their work on May 10, 1906. Later they applied the complement fixation test to a large number of cases of syphilis in Neisser's clinic. Aqueous extracts of syphilitic liver, placenta, glands, chancres, and gummata were used as antigens. Of 257 cases in all stages of the disease, only 49 reacted positively. Obviously, this small percentage of positive results is an indication of the poor technic originally employed. It was originally thought by Wassermann and his co-workers that the antigen in this reaction must be made from syphilitic material, but later work showed this to be erroneous. In 1907, Weygandt reported that he had obtained a positive reaction in tabes with an extract of normal spleen. Later in 1907, Landsteiner[4] and others showed that an alcoholic extract of guinea pig heart yielded results equal to those

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*Ann. Inst. Pasteur **15**: 289, 1901.

[1] Ann. Inst. Pasteur **16**: 734, 1902.

[2] Compt. rend. Soc. de biol **53**: 841, 1901.

[3] Deutsch. med. Wchnschr. **32**: 745, 1906.

[4] Wien. klin. Wchnschr. **20**: 1421, 1565, 1907.

obtained with an aqueous extract of syphilitic liver. These results indicated that the antigenic principle was soluble in alcohol, and a prolonged series of investigations on the various lipoids and their relation to the reaction was begun. This work apparently upset the theory of the Wassermann reaction. Modern work, however, shows that while Wassermann's original idea of the mechanism was incorrect, there is no doubt about the specific nature of the reaction. We believe now that the reaction is dependent upon a particular antibody, and that these can be demonstrated by suitable lipoidal substances. The method is diagnostic of syphilis, with the exception of yaws (see Chapter X). Assuming that yaws and syphilis are identical diseases, certainly the Wassermann reaction and its modifications are diagnostic of syphilis.

It has been affirmed and denied that the Wassermann test is specific, that it is found positive in diseases other than syphilis, notably malaria.

A complete study of the question of the Wassermann test in giving rise to nonspecific positive reaction was made by Saunders and Turner.[2] Sutherland and Mitra,[3] Di Natale,[4] Lloyd,[5] Fischer and Gunsberger[6] and Curth[7] in their writings suggested that malaria alone might cause a positive or partly positive Wassermann reaction. On the other hand, Johnson,[8] Iyengar,[9] Clark,[10] and Kolmer[11] indicated that malaria alone does not cause positive reactions. Altounyan[12] contended that acute malaria had an undoubted effect in increasing the anticomplementary powers of the serum.

Their report is based upon the findings among a total of about 2,000 persons studied carefully in a yaws clinic in Jamaica in 1932. Seventeen per cent of these people were listed as having malaria.

Some important work on this subject was reported by Saunders and Bianco,[*] based upon observations made at the U. S. Naval Hospital at Bethesda, Maryland, during the year 1945. Serologic tests were recorded at least once on all malaria patients admitted to the Tropical Disease Service of that hospital, and results of tests made on 411 consecutive admissions to the Service were analyzed. Most of the patients were admitted with acute malaria in the first few days of the attack, but a number were seen during remissions, several weeks after the last febrile attack. Some had as many as two to twenty-five attacks. They were all cases of vivax malaria. Positive or doubtful[†] Kahn reactions were found in 10.4 per cent. Some cases showed negative[‡] results with the first samples and positive results with the second or third sampling. Of 508 separate samples of blood examined, 43, or 8.5 per cent, gave doubtful or positive serologic reactions.

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[*] Kuba, June, 1947. Lab. Digest **11**: Oct., 1947.

[†] Reactive or weakly reactive.

[‡] Nonreactive.

[2] South. M. J. **28**: 542, 1935.

[3] Indian J. M. Res. **2**: 984, 1915.

[4] Revista di Malariologia **9**: 741, 1930.

[5] Ind. Med. Gaz. **47**: 1, 1932.

[6] Ztschr. f. Immunitätsforsch. u. exper. Therap. **78**: 295, 1933.

[7] Am. J. Syph. **17**: 164, 1933.

[8] J. Path. & Bact. **24**: 145, 1921.

[9] Indian J. M. Res. **8**: 136, 1920.

[10] Twelfth Annual Report, Medical Department, United Fruit Company, pp. 147-161, 1923.

[11] J. A. M. A. **93**: 1429, 1929.

[12] Lancet **1**: 73, 1924.

All reports indicate that malaria profoundly influences the serologic test for syphilis. A diagnosis of syphilis should not be made without eliminating the possibility of a concurrent malarial infection. This precaution becomes more important because of the large number of veterans who have contracted malaria overseas, especially since many are required to have premarital serologic tests to detect syphilis.

Conclusions by Saunders and Bianco were as follows:

1. Serologic tests for syphilis are frequently positive in nonsyphilitic persons following an attack of malaria.
2. Some studies indicate that when a variety of tests are made at intervals during and after an attack, one or more positive reactions can be demonstrated in every case.
3. The proportion of cases giving positive tests appears to be greater when clinical activity of malaria has persisted for more than a week.
4. The largest number of positive reactions appears first during the second to fourth weeks following onset of acute malaria.
5. Positive serologic tests resulting from malaria revert to negative in most cases within four weeks and in practically all cases within ten to twelve weeks following the acute attack.
6. Of 411 presumably nonsyphilitic malaria cases, doubtful or positive serologic tests were found in more than 10 per cent. Tests became positive for the first time in 10 per cent during the first week, 21 per cent during the second week, and 5 per cent during the third week, but a total of 29 per cent gave positive reactions during the second week, and 24 per cent during the third week following the onset of acute malaria.

The question of positive Wassermann or positive flocculation test in malaria in nonsyphilitic persons has been investigated by Burney, Mays, and Iskrant.[1] They selected eleven patients with functional psychoses. Syphilis was excluded by means of history, physical examination, two negative blood serologic tests, and one negative spinal fluid. Four controls were selected, two with syphilis and two without. The laboratories of Eagle, Hinton, Kahn, Kline, and Kolmer performed the tests on all specimens collected in the course of this study. Three patients were inoculated with 10 c.c. of blood containing *Plasmodium vivax*. The other eight patients were infected directly by the bite of an infected mosquito. The infected blood was obtained from functional psychotics with malaria who did not have syphilis as proved by negative history, physical examination, and blood and spinal fluid serologic reactions. Inasmuch as there is no evidence that the mosquito may transmit the spirochete of syphilis, it can reasonably be stated that these patients were inoculated only with *Plasmodium vivax*. Enough blood was withdrawn from each patient to send a comparable amount to each laboratory in the form of whole blood. Of these eleven patients, all cases had at least one positive reaction to the Kahn standard, and the Kline diagnostic tests. Our of eleven cases, positives were returned on nine according to the Kline diagnostic; one case returned three doubtfuls; and one case was never positive or doubtful. Out of ten cases the

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[1] Am. J. Pub. Health 32: 39, 1942.

Kolmer (Mahoney) complement-fixation test was positive on nine, and the other patient had one doubtful reaction. Only seven cases were positive to the Kolmer (Kolmer) complement fixation, and four cases were never positive. Five cases were positive out of eleven according to the Eagle Wassermann, and of the six that were never positive, one had a doubtful reaction. Only four patients out of eleven tested according to the Eagle microflocculation test were positive, and one of the other seven had two doubtful reactions. None of the ten patients were positive to the Hinton test, but three of them showed one doubtful reaction. According to this investigation, the duration of clinical activity, highest temperature reached, time of withdrawal of blood in relation to an individual paroxysm, or density of parasites, did not seriously influence the serologic reactivity of the blood. It is the opinion of these workers that, from the evidence presented, a diagnosis of asymptomatic syphilis based upon positive serology alone should not be made in an area where malaria is endemic without first eliminating the possibility of coincident malarial infection. This work seems to prove that malarial infections may give rise to positive complement fixation and flocculation tests.

In the years that have elapsed since the announcement of Wassermann and his coworkers, much work has been done in the direction of standardization of technic. There have been many discussions as to just what kind of antigen should be used, whether the patient's serum should be active or inactive, just how much complement should be used, etc. Out of this wealth of literature have come the standard methods which are discussed in this chapter.

We now believe that a *positive* Wassermann reaction is rather good evidence for the existence of a syphilitic infection, but that a *negative* reaction does not by any means exclude the probability of syphilis. We know that the reagin of syphilis is inconstant in any given case for any given period of time. We know that in primary syphilis a certain degree of tissue change must occur before the specific reagin appears in the blood. We do not expect the presence of a syphilitic reagin in the primary stage before the tenth day, after which time we are likely to find a positive reaction. In the primary latent period, that is, when the chancre is healing or in the pre-eruptive stage, a positive reaction is obtained in about 92 per cent of the cases. In the secondary, or eruptive stage, positive reaction is obtained in 100 per cent of the cases. In the latent stage of syphilis after the secondary symptoms have subsided, our experience has been that possibly 80 per cent of all syphilitics show a positive Wassermann. In the tertiary stage, about 90 per cent of all individuals show a positive Wassermann reaction. In the tertiary stage with lesions of the central nervous system, we find, in general paresis of the insane, from 90 to 100 per cent positives in the blood serum and nearly 100 per cent positives in the spinal fluid. In tabes, our personal experience has been about 60 per cent positives in the blood serum and about 90 per cent in the spinal fluid. In the active prenatal or first year, we find practically 100 per cent of congenital syphilitics show positive Wassermann reactions, and in the second year, about 80 per cent.

The cerebrospinal fluid of individuals with primary syphilis is usually negative. The cerebrospinal fluid, according to Wile, in secondary syphilitics gives a positive reaction in about 30 per cent of all cases. It is probably in tertiary syphilis that the Wassermann reaction has its greatest value. In untreated and active tertiary syphilis, the reaction is positive in about 95 per cent of all cases.

The effect of treatment on the Wassermann test has been much discussed. It is the *last* symptom to disappear under treatment and the *first* to reappear if complete sterilization has not been accomplished. In some cases, the Wassermann reaction seems to disappear with a rather moderate amount of treatment. This is, of course, no proof that the individual has been cured. A single negative reaction of the blood serum following the institution of treatment is simply evidence that the treatment has probably been partially successful, but certainly is no criterion of the probable outcome of the case. Only a succession of negative Wassermann reactions over a long period of time with a knowledge of the institution and amount of treatment is of advantage in drawing conclusions as to the probable results of the treatment. Successive examinations must be made over a period of at least two years and off and on during the remainder of life. Questions are frequently asked by patients as to how many negative Wassermann tests they must have before they can be considered cured. The answer is practically no different from that given by the celebrated syphilologist, Fournier, who was once asked by a student just how he determined the patient was ''cured'' of syphilis: the answer of the master was, ''Treat the case for three years and have him return in twenty-five years. If he has no symptoms at that time, I consider that he has been cured.'' There are several facts which must be considered in summing up conclusions as to cure, the advantage of treatment, and why the reaction disappears quickly in one individual and not in another. There are different strains of spirochetes varying in virulence. There are different degrees of individual resistance to this disease. Assuming that we have a patient with a high degree of resistance and an infection of a mildly virulent strain of spirochetes, we should find mild symptoms and a rapid disappearance of the Wassermann reaction. Taking a patient with low resistance and an organism of high degree of virulence, we find a persistence of the Wassermann reaction and the onset of very severe symptoms. Again, there may be a strain which has a predilection for nervous tissue. This has been said by some authors to explain why some individuals have certain lesions of the nervous system and others never develop such lesions. It is rather interesting that many cases of syphilis of the nervous system, such as paresis, are recruited from the more intelligent ranks. Is it possible that these individuals are prone to this infection because of their more highly developed nervous systems, which are more susceptible because of this higher organization?

Blood and Spinal Fluid Tests for Reagin After Treatment of Neurosyphilis

Clinicians are frequently disturbed by the persistence of a positive test for reagin after long-continued and persistent treatment. This is particularly true with respect to neurosyphilis. This question has been thoroughly studied by Thomas.[1] His conclusions are based upon a follow-up of patients treated by malaria or penicillin, with data obtained in a 5- to 14-year period on 181 patients with neurosyphilis treated with malaria therapy, and a 5- to 8½-year follow-up of 213 patients treated with penicillin. It was found that positive results from serologic tests for syphilis persisted in 80 to 84 per cent of patients followed for five years, and in 70 per cent of patients followed for ten years after treatment. Positive results from tests for spinal fluid reagin were found in 63 per cent of patients followed for five years, and in 27.7 per cent of patients followed for ten years after treatment. It is futile to re-treat patients solely because of the persistence of reagin in blood serum and spinal fluid. In cases in which treatment was given to note the effect on high titers in blood serum and spinal fluid reagin, no appreciable effect in titers was obtained (Thomas).

One must remember that reagin in blood serum is a complex substance consisting of more than one presumed antibody, and that factors other than syphilis may influence the production of one or more of these ''antibodies'' in known syphilitic patients as well as in persons who have biologically false positive results in tests for syphilis. One must also consider that relapses in neurosyphilis, as determined by increases in spinal fluid cell count and in results of other quantitative spinal fluid tests, never occur later than two years after treatment has inactivated the infection.

It is obvious, in the light of our present knowledge, that erroneous conclusions must not be drawn about a persistent positive test for reagin in either blood serum or spinal fluid. Unnecessary treatment of these positive persons should be avoided.

We find, too, a certain type of infection which is ''Wassermann-fast''; that is, no matter how much treatment is given, the Wassermann remains positive. No explanation has been given in the literature for this condition. Some have claimed that the Wassermann reaction is an indicator of resistance and that these individuals have a high degree of resistance. No facts of an experimental nature have been brought forward to explain this. It is paradoxical that at times syphilitic treatment may convert a negatively reacting serum into a positive one. It is also true that some cases of latent syphilis which give a negative reaction at first will become positive under the influence of injection of mercury or salvarsan. At times, attempts are made to utilize this phenomenon in the form of the *provocative* Wassermann; that is, for the purpose of confirming the diagnosis of syphilis where previously a negative reaction would seem to exclude it. Our experience with this method has been disappointing.

Craig and others have reported that the *ingestion of alcohol* by a patient with a positive Wassermann will tend to diminish the reaction or cause it to disappear. Our experience does not confirm this statement. In a general way, we can state that the Wassermann reaction serves two important purposes: first, it is an invaluable aid in the diagnosis; and second, it is a guide in the treatment of syphilis. Negative reactions do not exclude syphilis, but positive reactions include it. In all cases where any suspicion exists as to the presence of syphilis of the central nervous system, a spinal puncture and an examination of the spinal fluid should be performed. A positive reaction occurring in women about to abort is an indication for treatment and may protect the fetus. A positive reaction in either parent of a seemingly healthy infant is an indication for treatment of the child, especially if the mother reacts positively. Another point which must be borne in mind is that although a positive reaction indicates that the patient is syphilitic, it does not necessarily mean that a particular lesion is syphilitic. For instance, we had a very interesting case of a lesion of the tongue in which we obtained a four-plus positive Wassermann. This was in the early days of the Wassermann test before we had had an opportunity to weigh it in the balance after years of experience. Immediate treatment by injections of arsphenamine clearly stimulated the growth of the cancer of the tongue, for such it was, and in a few months the patient died from this condition.

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[1] J. A. M. A. 153: 718, 1953.

Just how long the reaction may remain positive after the patient is cured and all spirochetes are dead, of course, is a matter of conjecture. Experimental studies on rabbits and monkeys have shown that the reagin disappears very completely and quickly under therapeutic control. In a general way, it can be stated that a positive reaction is of great value, while a negative reaction is of comparatively little value in clinical medicine.

In regard to the *comparative value of the various tests* for syphilis, in other words, the *complement fixation* and the *precipitation methods,* our own opinion inclines strongly to the belief that the complement fixation methods are superior to the precipitation methods. We therefore urge that the complement fixation methods be continued as the methods of choice, rather than that they be abandoned, as some have already done. We are ready to admit, however, that precipitation methods are simpler and easier to control. They seem to yield more false positives than do complement fixation methods.

Summary: A Negative or Nonreactive Report.—A negative or nonreactive report is evidence that the patient has not active syphilis but does not mean that he never has had syphilis or that syphilis is not present at this time. It may also mean that he has a syphilitic infection too recently acquired to produce the substances which give the positive reaction.

The Weakly Reactive or Doubtful Reaction.—No weakly reactive test should be used as a diagnostic measure or as a measure of control of treatment. A borderline reaction is a finger of suspicion only, pointing at the individual, and must be verified and checked over by repetitions. In this connection, the provocative treatment by injection of arsphenamine may be attempted, testing the blood on the second and seventh days after the injection. If the reaction is still borderline, or negative, in a negative sense we may be emboldened further in our belief that syphilis is not present. Our experience with this has been disappointing.

The Positive or Reactive Reaction.—This is a very positive indication of syphilis. If the clinical symptoms are at variance with the positive serologic findings, this by no means eliminates the question of syphilis. Syphilis may be coincidental with other diseases, such as malignancy or tuberculosis. Positive Wassermann tests indicate tissue response to syphilitic infection. The cerebrospinal fluid is positive in all cases of general paresis and in about 60 per cent of tabes. Other writers give figures as high as 80 per cent for tabetics. In latent syphilis, the spinal fluid may show a positive reaction while the blood Wassermann is negative. So far as a positive reaction in tuberculosis is concerned, if this occurs, it indicates a coexistent disease with syphilis.

If possible, the positiveness or negativeness of Wassermann reactions should not be discussed with patients because of the difficulty of explanation of all these facts. In order to get good results in any case, the patient should have perfect confidence in his medical adviser. It is foolhardy for him to discuss serum reactions with patients where a medical training is vital to a perfect understanding of the facts. These patients should be told, of course, all the facts about the diagnosis and their manner of life under treatment, but under no circumstances should any discussion of the vagaries of the serum reaction be undertaken with the interested patient.

No attempt should be made to make any statement as to how long a patient should be treated before he is considered well, nor how many negative Wassermanns should be obtained before he is considered cured. If, after a lapse of several years of treatment, the Wassermann test is negative, and if, year after year, it remains negative, one might tell the patient that each negative which he obtains is better and better evidence of the probability of cure. No statement stronger than this should be made to the patient.

Reporting Results of Serologic Tests

It was recommended in the 1953 report of the National Serology Advisory Council to the Surgeon General of the U. S. Public Health Service that the following terms be used in reporting the results of serologic tests: *Reactive* instead of positive, *weakly reactive* in place of weakly positive or doubtful, and *nonreactive* to replace negative.

When reporting results of quantitative reactions, the end-point titer should be reported in terms of the greatest dilution in which the tested specimen produces a reactive (positive) result, and that the term "dils," a contraction of "dilution," be used to identify these dilution reactivity end points.

The Biologic False Positive Reaction for Syphilis

Refer to the treponemal immobilization test, page 2085.

Out of the many contributions which have been published on this point is an excellent article written by Stokes, Boerner, Hitchens, and Nemser.[1] This report gave the result of the fourteen-month period spent in studying the problem of the nonspecific positive, so-called "biologic false positive" reaction for syphilis. The bloods of Red Cross donors at the Philadelphia Blood Donor Center supplied the material. In all, 210,261 blood specimens were tested in a nine months' period and 79 of the donors, showing definite positive reactions, were subjected to subsequent clinical and laboratory examinations. Of 210,261 Red Cross donor blood specimens, 489 gave definite positive reactions to serologic tests for syphilis. This incidence of 0.23 per cent positives is substantially below even the low reported prevalence rates for the disease (0.5 per cent) in the Northwestern and Northeastern rural sections of this country as reported by serologic surveys. In this evaluation, the diagnosis of syphilis was reached in three months in 69.2 per cent; in nonspecific positive cases in only 22 per cent. Of the nonsyphilitic positive cases, 78 per cent required more than three months for decision. Only 40.5 per cent of the Red Cross donors with syphilis-positive bloods were finally adjudged to have syphilis. The remainder, 59.5 per cent, were finally adjudged to have actual or probable biologic false or nonspecific positive reactions.

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[1] J. A. M. A. 130: 57, 1946.

In evaluating this work, it was found that the clinician's history and examination of this group as a means of differentiating syphilis from nonsyphilis was, in contrast to its usual claims to ''court of last resort'' status, disappointing. The final resort was perforce too often serologic. With respect to history, there was a slight preponderance of recent vaccination, upper respiratory infection, inoculations, recent febrile illness, and infection in nonspecific positive cases. In the physical examination for ''landmarks'' of syphilis and ''suspicion arousers,'' the yield of definite findings was small, though better than from the history.

There was a fertile field of disagreement among both examiners and consultants about questions of physical examination—opinion on the level of the liver, presence or absence of true enlargement, the configuration of teeth and bones, the shape and height of the palate, the interpretation of pupillary reactions, and the interpretation of murmurs over the precordium. In the physical examination, evidence for the presence of syphilis was found largely in examination of the eyes, including slit lamp study for vascularization of the corneas, characteristic teeth in congenital syphilis, the skeletal changes of congenital syphilis, the facies, and the reflexes.

Special studies were made in response to apparent indications in the individual case but these had a rather small positive yield. The high points in the examination were spinal fluid examinations, heterophile antibody tests, radiographic examinations of the chest, and examinations for congenital stigmas.

The serologic studies devised for the purpose of this investigation were the Boerner classification procedure, an interpretation of simultaneously performed, exceptionally sensitive tests for the detection of flocculating and complement fixing reagin.

These workers found that all of the precipitation tests show quite high proportions of strong positives and of strong and moderate positives obtained in nonspecific blood sera. The Kolmer complement fixation test, as performed in Mahoney's laboratory, on the other hand, while less sensitive in the detection of syphilis, is a notably safer resort than any of the precipitation tests except the Kline diagnostic for the avoidance of strong positives in the nonspecific field. Broadly speaking, one would be inclined to infer that a precipitation test should either not be used or should be paralleled by a dependable and sensitive complement fixation procedure in any and every serologic survey of a mass material in which nonspecific positives may appear.

This study does not solve all of the difficulties intrinsic in both the identification and the exclusion of syphilis in any individual case. It indicates that a disconcerting serologic proportion obtained by a routine test procedure on a large scale sampling of blood in a population group will yield a high proportion of nonspecific positives. Many people have been subjected to treatment for nonsyphilitic or, rather, nonexistent syphilitic infection. The diagnosis of syphilis by history and physical examination is difficult to make as the infection passes into latency and even into the period of late manifestations. Therefore, recourse must be had to serologic procedure. The clinician may expect to identify indubitable symptoms and signs of the disease in a proportion of cases of seropositiveness depending on the type of material with which he is dealing. Many times he will throw a weight of suspicion, disappointingly small and based on slender and debatable evidence, into the scales with the serologic test. He sometimes is compelled to diagnose syphilis on the basis of repeated nonspecific positive reactions. Stokes and associates stated that the outright diagnosis of a nonspecific positive at the present time is laboratory rather than clinical—a consistent weak or doubtful positive reaction, its decline to negative within a reasonable time, currently estimated in the literature as approximately three months. A suggestion but certainly not a presumption in favor of nonspecific positiveness, in the majority of cases such as Stokes and associates had, may be raised by a record of intercurrent or recently antecedent infection, especially of the upper respiratory tract, accompanied by fever. The special test procedures to determine the character and location of such infection will usually be applied too late to be of material value in sustaining a diagnosis of nonspecific positive reaction. They therefore, at best, establish little more than a suspicion-arousing type of reaction in the mind of the consultant, and the final decision again falls to the serologic test procedure and in the future, let us hope, to a specific physiochemical test for nonspecificity.

He further stated that the serologic warnings of nonspecificity are again (1) consistent weak or doubtful positiveness, punctuated with occasional high titer precipitation reactions, (2) serologic fluctuation and discord, i.e., disagreement between tests and among laboratories, and (3) negative complement fixation with positive flocculation reaction. For the simplified and yet increasingly accurate differentiation of syphilitic from nonspecific positive sera on repeated testing, by a procedure easily available to well-equipped serologic laboratories, this investigation offered the Boerner classification procedure as deserving of widespread trial even though it may presently be overtaken by a contribution from the physical chemists.

Our own experiences are in complete agreement with Stokes' conclusions. We, too, had several cases of nonspecific reaction referred to us during the American Red Cross campaign for blood plasma. We believe that routine tests for syphilis in the laboratory should consist of both the complement fixation and the precipitation tests. In cases where speed is essential, we have used the Kline test and found it very satisfactory.

Test for False Positive Serologic Reaction for Syphilis.—

In connection with the false positive serologic reaction for syphilis, Brown, Kolmer, and Lynch[2] reported the results of an investigation of a method whereby the reagin responsible for false positive reactions in the test for syphilis occurring in individuals with certain diseases could be inactivated or destroyed without inactivating or destroying the syphilis reagin.

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[2] Am. J. Syph., Gonor. & Ven. Dis. 29: 200, 1945.

Technic.—Treat the serum chemically by adding 0.1 c.c. of carefully titrated N/3 or N/4 sodium hydroxide solution to 1 c.c. of serum. Then inactivate at 55° C. and proceed with the test.

Results were given following the application of this method to normal rabbit serum and that of nonsyphilitic human beings with malaria, infectious mononucleosis, leprosy, virus pneumonia, and certain other diseases, as well as to sera of patients with syphilis.

The method proved effective in preventing biologic falsely positive Kolmer, Kahn, and Mazzini reactions in sera of normal rabbits and in a large percentage of sera of presumably nonsyphilitic individuals with malaria and infectious mononucleosis. In each of these sera, N/3 sodium hydroxide was more effective than the N/4 solution. Less effective results were observed with the sera of presumably nonsyphilitic lepers, and in leprosy the N/4 solution was found to produce results superior to those following the use of N/3 sodium hydroxide.

It was found that this treatment applied to syphilitic sera results in the inactivation or destruction of some syphilis reagin, but according to Brown and associates, falsely negative Kolmer complement fixation reactions are likely to occur only in the case of sera containing small amounts of reagin.

Furthermore, the addition of N/3 or N/4 sodium hydroxide solution to rabbit and human sera has prevented the occurrence of anticomplementary reactions in the Kolmer complement fixation test. There was some loss of syphilis reagin, but again the authors noted that falsely positive negative reactions are not likely to occur except in the case of sera containing small amounts of reagin.

In Venereal Disease Information for March, 1945, further consideration was given to this question of biologic false positive serologic reactions for syphilis. Such false positives are common in leprosy, malaria, infectious mononucleosis, vaccination against smallpox, rat-bite fever, relapsing fever, lupus erythematosus, and possibly atypical pneumonia. There is no reliable evidence that the tests are affected by pregnancy, menstruation, scarlet fever, jaundice, subacute bacterial endocarditis, tuberculosis, or hyperproteinemia. Inadequate data are available on measles, mumps, infectious hepatitis, lymphogranuloma venereum, and chancroid.

In this communication it was stated that transient false positive reactions may occur in apparently normal individuals without recent illness and in cases of some diseases the incidence of which is so low as to suggest that the relationship may be coincidental. It has been suggested that even persistently positive reactions may occur in nonsyphilitic patients. In widespread serologic surveys the number of innocent victims may be large.

Since low titer syphilitic sera may show discrepancies between the results obtained with various test antigens and fluctuation in apparent reactivity of successive sera, these are not adequate criteria for considering a positive reaction true or false.

The various complement fixation and flocculation tests for syphilis are antigen-antibody reactions. The lipid antigen is widely distributed in human and other mammalian tissues, and is an effective antigen for forming antibodies if mixed with a foreign protein before inoculation. It is not clear whether the Wassermann antibody is formed in response to Wassermann antigen from the organisms or to tissue antigen rendered active by the spirochete. If the latter hypothesis is correct, no difference would be expected to exist between the Wassermann antibodies in syphilis and in other diseases.

Some of the false positive reactions may be eliminated in the future by purification and improvement in specificity of lipid antigens of the Wassermann group, but there is no reason to expect all false Wassermann antibodies to differ in any given respect from the true antibody. Attempts to find consistent empirical physicochemical differences betwen syphilitic and false positive sera have thus far failed. The most promising approach to the problem is the detection of antibodies to antigens of the spirochete other than Wassermann antigen. See the treponemal immobilization test, page 2085.

7. Hemolysis

By "hemolysis" is meant the digestion or dissolution of blood cells by various agents. Hemolysis may be caused by chemical or bacteriologic factors. For instance, blood cells are hemolyzed by the addition of distilled water and mineral acids. A specific blood cell, for example the sheep cell, would be hemolyzed or digested in the presence of the specific antisheep hemolysin and sufficient complement. Excessive or prolonged heat, toxins of certain bacteria, undue agitation, age, and other factors cause hemolysis of blood cells.

8. Hemolysin or Amboceptor

Hemolysin is a substance, either present normally, or artificially produced, in the blood serum of animals, that is capable of dissolving the blood cells against which it has a specific action. This reaction does not take place unless complement is used. Hemolysin is artificially produced by the injection of blood cells of one species of animal into the blood stream of another animal, at definite intervals. The blood serum of the animal injected contains the hemolysin which is specific in dissolving the blood cells of the species of animal the cells of which were used for injection.

For example, if sheep cells are injected into the blood stream of a rabbit, the rabbit blood serum develops antisheep hemolysin. This hemolysin is capable of dissolving sheep cells (if complement is present in the reaction). Any other type of hemolysin is produced in a similar fashion.

9. Sheep Cells

The washed blood cells of a sheep are used in complement fixation tests if antisheep hemolysin is used. If antichicken hemolysin is used, chicken cells are used in the test.

10. Hemolytic Index

Hemolytic index refers to the amount of hemolytic action (or blood cell digestion) the blood serum has naturally against a given species of blood cells. For example, if 0.1 c.c. unheated patient's serum is capable of causing hemolysis of 0.8 c.c. sheep cell suspension (without the addition of hemolysin or complement from another animal) the hemolytic index is 0.8 c.c. This term is used particularly in the Hecht-Gradwohl test.

11. Anaphylaxis (see page 1339)

Anaphylaxis is shock produced by the injection of a foreign protein into an animal whose tissues are sensitized to the protein injected. It may be natural or acquired. It is produced by injecting the foreign protein the second time into an animal after a period of time long enough to permit sensitization to develop.

12. Antigen-Antibody Reaction

An antigen-antibody reaction is the reaction which takes place between an antibody and a specific antigen. In the Wassermann reaction, it is seen in the production of complement fixation. The reaction in the Wassermann test for syphilis does not utilize a true antigen (that is, an extract of *Treponema pallidum*) but is an extract of lipoidal substances. A true antigen-antibody reaction is illustrated in the tuberculosis complement fixation test where the blood serum contains the true antibody of tuberculosis, and the antigen is made by extracting tubercle bacilli.

13. Reagin

The term ''reagin,'' originated by Coca, is employed to designate what in respect to other microbic diseases would be called the ''antibody of syphilis.'' No one has found antispirochetal antibodies in the blood sera of syphilitic patients. Syphilitic serum does not agglutinate the spirochetes in material from lesions and does not contain treponemacidal substances. Syphilitic serum does not agglutinate cultures of *Treponema pallidum.* Rabbits injected with these cultures develop agglutinins for the culture spirochetes but these sera have no protective action when injected into rabbits along with virulent organisms. It is generally believed that humoral antibodies have no significance in immunity in syphilis. Nevertheless, we speak of ''antigen-antibody'' reactions in syphilis purely in a theoretical sense. The term is incorrect in view of the foregoing statements, but is used purely in an artificial sense.

14. Anticomplementary

The meaning of the term ''anticomplementary'' is as follows: any condition which arises in a complement fixation test which, by itself, causes destruction of complement. A serum may **become** anticomplementary, or it may be naturally anticomplementary. It becomes anticomplementary through age. bacterial contamination, or other unknown factors.

15. Thermolabile

Any substance which is affected or destroyed by heat is thermolabile. Complement, for example, is thermolabile.

16. Thermostable

Any substance which is unaffected by heat. Hemolysin, for instance, is thermostable.

17. Hypertonic

A hypertonic solution is one that contains an excessive amount of chloride over the physiologic amount, 0.85 per cent, or which has a higher osmotic pressure than the blood.

18. Hypotonic

A hypotonic solution is one that contains chloride under the physiologic amount, 0.85 per cent.

19. Cholesterinization

Cholesterinization is the rendering of an antigen more sensitive by the addition of a small amount of cholesterol.

THE WASSERMANN TEST OF BLOOD

Preparation of Glassware

New glassware should be immersed in a freshly prepared weak solution (1:1,000) of nitric acid overnight. The following day, rinse thoroughly in tap water to remove the acid. Rinse thoroughly in distilled water. Wrap in paper, and sterilize in the dry heat oven at 170° C. for two hours.

After use, the glassware should be rinsed thoroughly to remove any blood or serum that may adhere to the glass, then wrapped and sterilized.

Do not allow the pipettes or tubes to become brownish. This can be avoided by removing all traces of blood serum or blood corpuscles from the glassware before sterilizing.

Do not use soap or strong chemicals, as it may produce artificial hemolysis of the blood during the performance of the test.

The glassware should be cooled to room temperature before use.

All glassware used in the Wassermann test, including the graduated cylinders, must be clean, sterile, and dry. If glassware shows even a trace of water, it should not be used.

Patient's Serum—Method of Venepuncture

There are certain details about venepuncture which should be borne in mind. The same principles apply whether we are obtaining blood for bacteriologic or serologic purposes. It is first necessary to find a suitable vein on the arm, usually the median basilic vein where it crosses the anterior flexus of the elbow. In babies, we have used the jugular vein of the neck or have simply plunged a large needle into the heel. The patient should be placed in a comfortable position, preferably lying down on the operating table. This facilitates ease of puncture and prevents shock. The instrumentarium should be either the 19-gauge needle described below or a needle attached to a glass syringe. Necessarily, both syringe and needle should be previously sterilized at the proper dry heat sterilization temperature, 170° C. for two hours. Sterilization by boiling is objectionable because it is desired not to have any water in contact with the specimen of blood which is obtained.

With the vein selected and with the arm placed in a comfortable position (on the side arm connected to the operating table) and covered with a sterile towel, one prepares the skin by rubbing with tincture of iodine. Remember that fairly fresh tincture of iodine should be used because it tends to concentrate when it becomes old or if the stopper of the bottle is left out. This concentration is likely to burn the skin of the patient. We now apply a tourniquet; either a flat band of rubber may be used or, preferably, the cuff of a blood pressure apparatus. The cuff of the blood pressure apparatus should be adjusted to the upper arm and applied so as just about to hear the diastolic pressure around 90 to 100. After a deep mahogany color is produced by the application of the iodine, a minute or two is allowed, and then the puncture is started. The selection of the needle is important. One with a rather short bevel is preferred. Be sure that the stylet has been moved back and forward in the needle so that there is no obstruction. Insert the needle almost parallel to the skin. The tendency of most beginners is to plunge the needle directly downward, which perforates both anterior and posterior walls, fails to get blood, and produces an objectionable hematoma in the surrounding tissue. The technic of entering the vein is very much like that of picking up a stitch in a piece of goods, namely, sliding along the anterior wall with a slight dipping motion, penetrating only the anterior wall. The process should be rather swiftly performed so as to produce the minimum of pain. The pain is practically negligible provided the needle is sharp and the operator is swift, and swiftness will come with experience.

Having obtained sufficient blood, the tourniquet or blood pressure cuff is rapidly removed and the needle is then withdrawn. Do not withdraw the needle before the pressure above is relieved, otherwise a hematoma is likely to occur. A pledget of alcohol-soaked cotton is held in the hand and pressed down over the puncture point while the needle is withdrawn.

After the needle is removed, cover the puncture with a small piece of adhesive tape.

If the blood is to be used for serologic work, expel it into a sterile test tube and allow it to clot. If it is to be used for bacteriologic purposes, it should be ejected into a sterile flask or large sterile test tube with a tightly fitting sterile cork and defibrinated to prevent clotting. If it is to be used for serologic purposes immediately, place the clotted blood in the incubator and wait a few minutes before centrifugalization. If it is not to be used until later, place in refrigerator.

Fig. 326 shows various types and sizes of needles used in laboratory procedures. Needles of gauges 19 and 20 are used for venepuncture; gauges 23 and 24 for subcutaneous injections; gauges 15 and 17 are useful in connection with obtaining blood for blood transfusion purposes or for use with the Baxter vacuum outfit.

Needles for taking the blood are the hose-type 19 gauge 1 inch needle (Becton-Dickinson Company). Place a wire in the needle, thrust the point into a cork test tube stopper and place the cork containing the needle into the test tube that is to be used for collecting the patient's blood. The whole is sterilized at 170° C. for two hours.

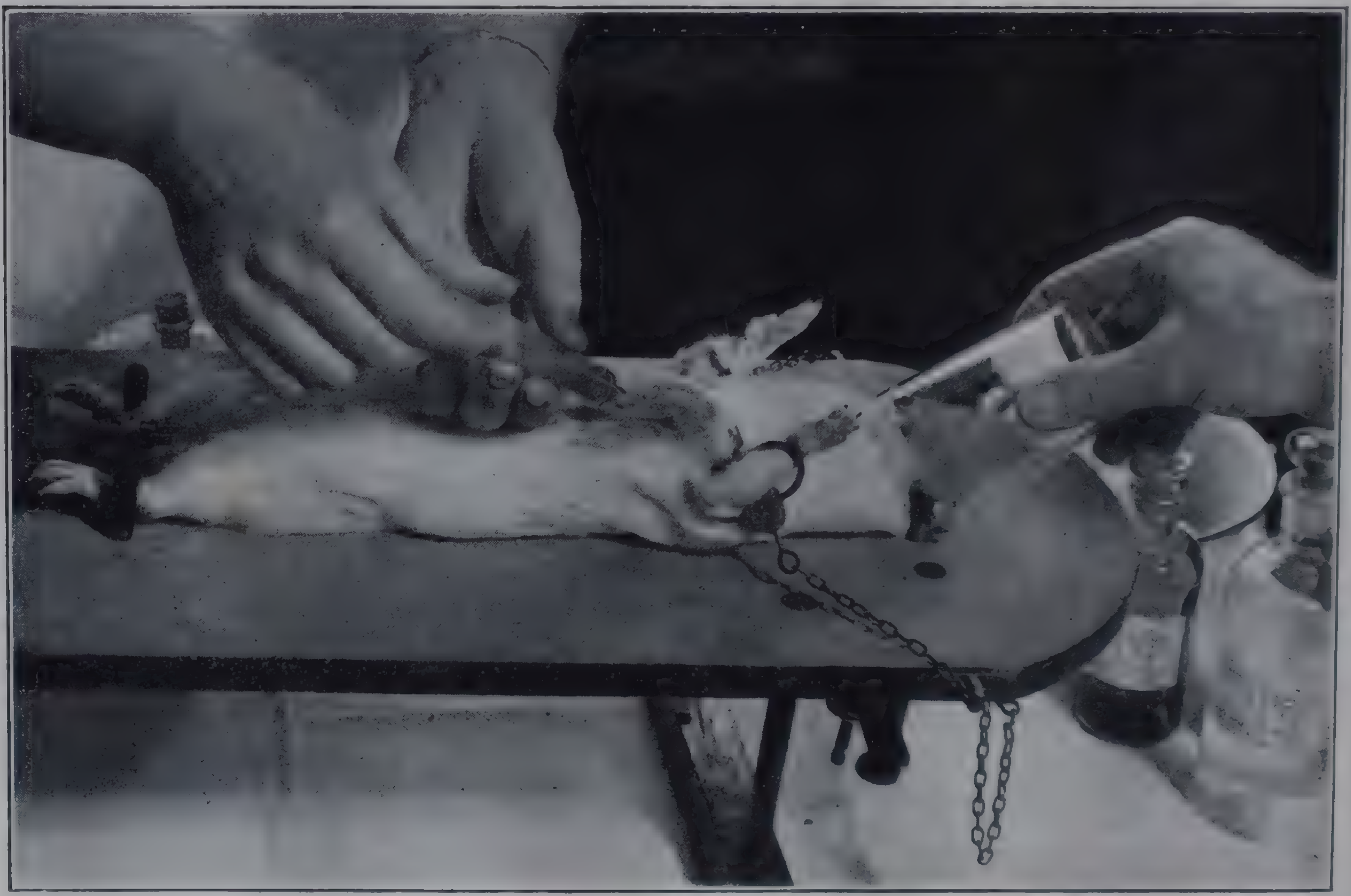

Fig. 594.—Withdrawing blood from the heart of a guinea pig. This is the Friedberger-Gradwohl apparatus. After the guinea pig is anesthetized, this is an excellent position for cardiac or carotid artery work.

Each blood, after it has been entered into the laboratory specimen book, and given a number, should be recorded on a sheet of paper representing the blood specimens of the day. After recording on the record sheet, loosen the clot from the side of the tube with a sterile wooden applicator. Centrifuge at high speed for 15 minutes to separate the serum from the clot.

Using a sterilized dropper, remove the clear supernatant fluid, placing it in a sterile test tube. If the serum is to be used for the Hecht-Gradwohl test, as well as for the Wassermann test, it is divided between two test tubes.

The serum for the Wassermann, Kahn, Kline, and Kolmer tests is placed in the inactivator at 56° C. for thirty minutes. The serum for the Hecht-Gradwohl test is ready for use; i.e., it is not heated.

It is customary to use letters of the alphabet as markers for the various sera in each day's work. The sera are then tested as A, B, C, etc.

When a serum has been tested, and is to be saved as a serum-control, a sterile cork stopper is placed in the tube, the tube labeled and dated, and placed in the refrigerator.

Physiologic Saline

This is 0.85% sodium chloride solution in water.

Dissolve 8.5 grams sodium chloride, c. p., in 1,000 c.c. distilled water in a large bottle.

The sodium chloride must be very accurately measured, and the quantity of distilled water must be exactly 1,000 c.c.

Mix thoroughly until the sodium chloride is dissolved and equally distributed throughout the solution by thoroughly shaking the large bottle.

Filter through filter paper into brown bottles of 500 c.c. capacity.

Fill these bottles about two-thirds full. Plug with nonabsorbent cotton. Cover with paper. Autoclave for sterilization at 15 pounds pressure for thirty minutes.

Do not use before the solution has cooled to room temperature.

Saline solutions should be made at least once, and preferably twice, a week.

If brown bottles are not used, keep the saline solution in a dark cabinet when not in use.

Preparation and Titration of Complement for the Wassermann Test

The blood serum of guinea pigs is used in the Wassermann test to supply complement.

Assemble the following materials:

Guinea pig and animal board.
Five c.c. Luer syringe with 1½ inch 21 gauge needle, sterilized in the dry heat oven at 170° C. for two hours.
Test tube for collecting the blood.
Sterile sponges.
Alcohol or iodine.
Ether anesthetic.
Sterile saline in test tubes for emergency injection, or for opening needle if it becomes plugged.

Place the animal on the animal board. (Fig. 594.)

Shave the skin over the area of the heart.

Locate the heartbeat (left ventricle) by palpation.

Give the animal a light anesthetic.

Disinfect the skin with alcohol or iodine.

Plunge the needle attached to the syringe into the heart, taking care that the needle is in the left ventricle.

Slowly withdraw 5 or 10 c.c. blood from the heart.

Eject the contents of the syringe into a sterile test tube.

Inject 5 c.c. saline into the abdominal wall of the guinea pig, and release it from the board.

Allow the blood to clot. Loosen the clot from the side of the tube with a sterile wooden applicator. Centrifuge at high speed to separate the serum from the clot. Remove the clear supernatant serum from the clot by means of a sterile dropper, placing it in a sterile test tube. If there are any blood cells in the serum, recentrifuge it and decant the cell-free serum.

The serum is now ready for dilution and titration preliminary to use in the Wassermann test.

Technic of Dilution and Titration of Complement for the Wassermann Test

Place 7 c.c. physiologic saline in a sterile cylinder. (This should be measured with a 10 c.c. graduated pipette.)

Add 1 c.c. guinea pig serum.

Dilute the hemolysin as for the Wassermann test.

Use the suspension of sheep cells which is to be used in the day's work.

Set up four Wassermann tubes in a rack and carry out the titration as in Table 186.

TABLE 186

TUBES	1	2	3	4
Saline	1.2 c.c.	1.1 c.c.	1.0 c.c.	0.9 c.c.
Complement	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep Cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Total Volumes	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.

Place in the water-bath 37°-40° C. for five minutes.

Accurate timing is important.

The saline is placed in each of the four tubes, then the complement, then the hemolysin, then the sheep cells.

After the tubes are ready for the water-bath, they are shaken thoroughly to mix the contents. The quantity in each tube should be the same.

At the end of five minutes' incubation, observe for hemolysis of the sheep cells.

If all tubes show hemolysis, 3 c.c. physiologic saline should be added to the diluted complement, and the titration repeated.

The tube containing the smallest amount of complement that shows complete hemolysis is the unit tube. Add 0.1 c.c. to the unit to find the titer for the day's work.

For example: Tube 1 shows no hemolysis.
Tubes 2, 3, and 4 show complete hemolysis.
Tube 2 contains the smallest amount of complement that will completely hemolyze 1 c.c. of diluted sheep cells. The amount in Tube 2 is 0.2 c.c.
0.2 c.c. + 0.1 c.c. = 0.3 c.c., or the titer of complement for the day's Wassermann tests.

If all four tubes show no hemolysis, either the complement is too weak, or the other reagents are not in perfect condition. If the complement is fresh, it is reasonable to assume that the fault is in the saline, hemolysin, or sheep cells.

If the saline is at fault from high concentration, a new solution will have to be made.

If the hemolysin is too weak, a stronger solution of hemolysin must be diluted, and another titration made.

If the sheep cells are in too heavy suspension, dilute further, and retitrate the complement.

Lack of hemolysis in any tube, when the complement is fresh, is frequently due to too weak a hemolysin.

If the mixture in Tube 1 is not hemolyzed, in Tube 2 is half or partially hemolyzed, and in Tube 3 is completely hemolyzed, we assume that the smallest amount of complement that will cause complete hemolysis is 0.25 c.c., the quantity midway between 0.2 c.c. and 0.3 c.c. The titer in such a case is 0.25 c.c. + 0.1 c.c., or 0.35 c.c.

If the first three tubes show no hemolysis, and Tube 4 shows complete hemolysis, we usually discard the complement for a fresh complement, as 0.5 c.c., the titer in this case, is too large a quantity to use in the test.

The titration of complement not only determines the volume of diluted guinea pig serum to be used in the test, but a successful titration is a check of the reagents, saline, hemolysin, and sheep cells which are also to be used in the day's Wassermann tests. This titration should always be carried out immediately before any Wassermann tests are made. No further check of these reagents is necessary.

Complement may be saved for several days, provided a titration is made prior to the Wassermann test. It usually deteriorates after two or three days. It should be kept in a corked container in the refrigerator.

The pooled blood serum of several guinea pigs is usually higher in complement than the blood serum of a single guinea pig.

The titration of complement is the same whether it is to be used in the Wassermann test, or in the titration of hemolysin or antigen.

If the titration of complement shows that the saline used was stronger than 0.85 per cent, all reagents will have to be rediluted with fresh saline before a test can be made.

NOGUCHI'S ETHER-SOLUBLE ACETONE-INSOLUBLE ANTIGEN

Preparation

Obtain beef heart and beef kidney from the abattoir.

Remove all fat and fibrous particles, cutting the meat into very small pieces so that there is no difficulty in finding and removing all undesirable material.

Grind in a meat grinder.

Add 50 grams beef heart, finely ground, and 50 grams beef kidney, finely ground, to 1,000 c.c. 95% ethyl alcohol.

Place all in a large bottle, stopper with a rubber stopper, shake thoroughly and place in the incubator at 37° C. for ten days, shaking twice a day during this period.

Filter through filter paper into a large, flat, clean, white porcelain pan.

Cover the pan with a thin layer of gauze to prevent contamination by flies or other insects.

Evaporate to dryness by means of an electric fan. This process may take from twenty-four hours to ninety-six hours.

A yellowish sticky residue remains in the pan.

Take up the residue in 75 c.c. ether. This is accomplished by marking a wide-mouth colorless bottle at 75 c.c., then adding ether to the pan, a small amount at a time, pouring into the bottle, adding more ether, etc., until all the residue has been dissolved or suspended in exactly 75 c.c. total quantity of ether.

Stopper the bottle tightly, holding the stopper in place with adhesive tape. Place in the refrigerator for twenty-four hours. The temperature of the refrigerator should be from 0 to 5° C. The insoluble substances will precipitate during the twenty-four hours.

Decant the supernatant fluid in which the ether-soluble substances of the meat are dissolved. Place the fluid in a casserole.

Evaporate the liquid in the casserole to 20 c.c. This can be judged approximately by placing 20 c.c. water in another casserole of the same dimensions, and comparing the two quantities.

Add 40 c.c. chemically pure acetone, free of methyl alcohol. (A very satisfactory acetone is Merck's reagent grade.) A precipitate will form immediately. This precipitate contains those lipoidal substances from the meat which are both soluble in ether and insoluble in acetone.

Cover the casserole with a thin layer of gauze, and allow it to remain at room temperature for an hour.

Pour off the supernatant fluid, keeping the sediment.

Place sediment in a wide test tube. Remove all traces of the acetone by pressure with a glass rod, pour off, leaving a sediment of a resinous consistency.

Cork the test tube tightly, label "stock antigen," and place in a cool box for at least two weeks to one month before diluting for titration.

This **Stock Antigen** can be kept indefinitely, unless it becomes contaminated with molds.
Prepare a **Stock Antigen Solution** from this stock antigen.

Stock Antigen Solution

Dissolve 0.3 gram stock antigen accurately measured on analytical balance

in 1.5 c.c. ether. The 0.5 c.c. ether is added to take care of loss by evaporation. When larger quantities of the stock dilution of antigen are made, it is not necessary to figure 0.5 c.c. for evaporation for each quantity.

Add 9.0 c.c. chemically pure methyl alcohol, free of acetone. (The methyl alcohol of the National Aniline and Chemical Company is excellent for this purpose.)

Mix thoroughly. A slight precipitate will form. The supernatant fluid is used.

To use in the Wassermann test, the antigen must be diluted with physiologic saline.

The exact dilution of each antigen varies but seldom.

From our experience in making hundreds of bottles of antigens in our laboratories, it has been found that in almost every case a dilution of 1 part of stock antigen solution with 9 c.c. physiologic saline gives a titer of 0.2 c.c. Nevertheless, each new quantity of antigen must be titrated before use. Since 0.2 c.c. is the titer of antigen that is desired, the titration of antigen is usually made first with a dilution of 1:10. Any subsequent dilutions of antigen are made if this dilution is insufficient, or if the exact strength of the antigen is desired.

Titration of Antigen

The dilution of the antigen may vary. It is, however, as previously stated, usually 1 part of antigen solution to 9 parts of physiologic saline.

In the titration of antigen, that quantity is used which gives (1) a strong (++++) positive reaction on a known positive (++++) serum when ½ the quantity to be used in the Wassermann test is employed; and (2) a clear negative reaction on a known negative serum when double the quantity to be used in the Wassermann test is used.

Example:

If 0.2 c.c. of antigen is to be used in the Wassermann test, 0.1 c.c. of antigen must give a ++++ positive reaction on a known ++++ positive serum; and 0.4 c.c. antigen must give a negative reaction on a known negative serum.

Prepare and titrate fresh complement.

Dilute the hemolysin and sheep cells.

Have on hand about 5 c.c. known ++++ positive serum and 5 c.c. known negative serum.

Make three dilutions of the stock antigen solution:

1:10, made by adding 1 c.c. stock antigen solution
to 9 c.c. physiologic saline
1:15, made by adding 1 c.c. stock antigen solution
to 14 c.c. physiologic saline
1:20, made by adding 1 c.c. stock antigen solution
to 19 c.c. physiologic saline.

The diluted antigen will be opalescent.

Carry out the titration of antigen according to the following chart, using each antigen dilution. It requires three Wassermann racks for the full titration.

Rack up the tubes so that there are four Wassermann tubes in the front row of holes at the left of the rack, and four at the right of the rack.

Place one tube behind the four tubes at the left, and one tube behind the four tubes at the right of the rack. The back row (two) of tubes are the serum controls. These are always used to be sure that the serum employed in the titration has not become anticomplementary.

TABLE 187.—TECHNIC OF TITRATION OF THE ANTIGEN WITH A KNOWN ++++ POSITIVE SERUM

TUBES	1	2	3	4	CONTROL
Known ++++ serum	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.
Saline	0.7 c.c.	0.6 c.c.	0.5 c.c.	0.4 c.c.	0.8 c.c.
Complement	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.
Antigen	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	------
Place in the water-bath at 37°-40° C. for 30 minutes.					
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Place in the water-bath at 37°-40° C. for 30 minutes.					
Total quantity	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.

TABLE 188.—TECHNIC OF TITRATION OF THE ANTIGEN WITH A KNOWN NEGATIVE SERUM

TUBES	1	2	3	4	CONTROL
Known negative serum	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.
Saline	0.6 c.c.	0.4 c.c.	0.2 c.c.	------	0.8 c.c.
Complement	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.
Antigen	0.2 c.c.	0.4 c.c.	0.6 c.c.	0.8 c.c.	------
Place in the water-bath at 37°-40° C. for 30 minutes.					
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Place in the water-bath at 37°-40° C. for 30 minutes.					
Total in each tube is	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.

The complement varies according to the previous titration result.

The quantity of saline in each tube varies according to the amount of complement used.

The control tubes should always show complete hemolysis.

If these tubes show no hemolysis, the serum used is anticomplementary, and another serum must be substituted.

Tubes 1 and 2 are the two tubes which are of importance if a titer of 0.2 c.c. is desired. If any other titer is preferred, Tubes 3 and 4 are the important tubes. In the titration with a known positive serum, if Tube 1 shows complete inhibition of hemolysis (cloudy tube with precipitate of cells), then 0.1 c.c., or ½ the desired titer, gives a strong positive reaction on a known ++++ positive serum. If this tube shows no hemolysis, the other tubes (2, 3, and 4) will show the same result.

If this tube shows complete, or partial hemolysis, the titer of the antigen cannot be 0.2 c.c.

If all tubes show complete hemolysis, the antigen must be discarded, as it is too weak in its action to be of any value in the Wassermann test.

In the titration with a known negative serum, Tube 2 contains 0.4 c.c. antigen, and is, therefore, the tube in which the titration result is determined. If this tube shows complete hemolysis, 0.2 c.c. antigen may be used as a titer, since double the quantity, or 0.4 c.c., gives a clear negative reaction on a known negative serum. The titer depends, obviously, on the result in the titration with a known positive serum also.

If all tubes show complete hemolysis, the antigen is such that the worker can be absolutely sure that no false positive reactions will occur. False positive reactions may be due to too strong an antigen.

If all tubes show complete, or partial, inhibition of hemolysis, the antigen must not be used in this dilution. If this occurs, the titration results with a 1:15, or a 1:20, dilution of the antigen must be studied for the purpose of determining the exact dilution of the antigen to be used in the subsequent Wassermann tests.

It is well, in the titration of antigen, to test its action on many known positive and negative sera before using it in the Wassermann tests. In this way, false reactions due to faulty antigens can be avoided.

The antigen is a very important reagent in the Wassermann test, and like all other reagents, great care must be used in its manufacture and titration.

In the preparation of any antigen for any complement fixation or precipitation test for syphilis, the titration should always be carried out on known positive and negative sera. Any other method of titration, if employed, is inadequate in judging the action of an antigen. In the titration just described, if ½ the titer used in the Wassermann gives a positive reaction on a strongly positive serum, it is reasonable to assume that the antigen is capable of detecting antibodies in weakly positive serums when 0.2 c.c. are used. The reaction on sera of this type are not false positives, since false positive reactions are guarded against by titrating against a negative serum, in which it is shown that quantities of antigen as high as 0.4 c.c., or in many cases, as high as 0.8 c.c., do not give positive results.

After antigen has been diluted in saline, it is not stable, and must be discarded after the Wassermann tests are made.

It is well to dilute only the quantity needed in the day's work. This is a definite saving of stock antigen.

Preparation of Cholesterinized Antigen from Noguchi's Ether-Soluble, Acetone-Insoluble Antigen

Make 10 c.c. **Stock Antigen Solution** (page 2035).

Add 100 mg. cholesterol, c. p., and dissolve.

If placed in the refrigerator, the cholesterol may precipitate. Before use, place in a water-bath at 37° C. until the cholesterol is again in solution.

When this antigen is diluted 1:10, the cholesterol present is in the proportion of 1:1,000.

Obviously, if the final dilution of the antigen with saline is to be 1:15, instead of 1:10, 150 mg. of cholesterol are used instead of 100 mg. Similarly, if the dilution is to be 1:20, 200 mg. cholesterol are added to the stock solution.

The cholesterol is added to the stock solution, since it is soluble in the methyl alcohol present but not in saline.

Preparation and Titration of Antisheep Hemolysin

Select large, healthy rabbits for injection purposes. The animals should be quarantined for several weeks before using until the condition of their health is established.

Use washed sheep cells for injection to prevent anaphylaxis. The sheep cells should be warmed to body temperature before use.

Use 1 c.c. tuberculin syringes so that the quantity of cells injected is accurately measured.

Remove the hair over the area of the marginal ear vein.

Disinfect the skin with alcohol.

Rub xylol into the skin to dilate the vein.

Select a point for the first injection near the tip of the ear; a point higher up or more proximal for the second; etc.

Inject 1 c.c. of washed sheep cells into the vein. See Figs. 597 and 598.

Tag the animal and record its number and the date of inoculation.

Repeat the injections at four-day intervals until 4 injections have been given.

Allow three days to elapse before withdrawing blood from the animal to test the amount of hemolysin produced.

Withdraw about 5 to 10 c.c. blood from the ear vein of the injected rabbit after the three-day interval has passed.

This is accomplished either by using a syringe to withdraw the blood from the marginal vein, prepared as above, or by making an incision into one of the ear veins, and allowing the blood to run into a test tube.

Allow the blood to clot.

Loosen the clot from the side of the tube with a sterile wooden applicator.

Centrifuge at high speed for fifteen minutes.

Decant the clear supernatant serum, placing it in a clean dry test tube.

Inactivate at 56° C. for thirty minutes to destroy the natural complement in the serum.

The quantity of hemolysin in the serum is then *roughly estimated* as follows:

Make the following dilutions of the rabbit blood serum:

1 drop of serum in 80 c.c. sterile saline (physiologic)
1 drop of serum in 100 c.c. sterile saline (physiologic)
1 drop of serum in 140 c.c. sterile saline (physiologic)
1 drop of serum in 160 c.c. sterile saline (physiologic)
1 drop of serum in 200 c.c. sterile saline (physiologic)
1 drop of serum in 250 c.c. sterile saline (physiologic)
1 drop of serum in 300 c.c. sterile saline (physiologic)
1 drop of serum in 400 c.c. sterile saline (physiologic)

Mix the serum dilutions very thoroughly, since the quantity of serum in saline is extremely small.

Measure the drops with a 1 c.c. pipette, holding the pipette in a horizontal position, and allow the drop to collect on the under side of the pipette.

The physiologic saline is added to the containers first. The serum is placed in each container with the same pipette to insure uniformity of the drop.

After the dilutions have been made, prepare the dilution of sheep cells in the same manner as for the Wassermann test.

Dilute and titrate the complement (which must be fresh), using for this titration a hemolysin which has previously been titrated, and the strength of which is known. After the titration of the complement, the diluted hemolysin used in it may be discarded.

Rack up 8 Wassermann test tubes.

Carry out the rough titration in Table 189.

TABLE 189

TUBES	1	2	3	4	5	6	7	8
Dilution of hemolysin	1 drop in 80 c.c. saline	1 drop in 100 c.c. saline	1 drop in 140 c.c. saline	1 drop in 160 c.c. saline	1 drop in 200 c.c. saline	1 drop in 250 c.c. saline	1 drop in 300 c.c. saline	1 drop in 400 c.c. saline
Saline	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Complement*	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Total quantity	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.

*The quantity of complement varies according to the previous titration results.

Place in the water bath at 37° to 40° C. for fifteen minutes.

The quantity of saline will vary according to the quantity of the complement, so that the total will remain 2.8 c.c. The saline is placed in the tubes first. The amount of saline is figured after the previous titration of complement.

Tube 1 received 0.5 c.c. hemolysin diluted 1 drop in 80 c.c. saline; Tube 2, 0.5 c.c. hemolysin diluted 1 drop in 100 c.c. saline, etc.

A different pipette is used for measuring each different quantity of hemolysin.

Shake the rack thoroughly before placing in the water-bath.

Observe at the end of every five minutes for hemolysis.

If no hemolysis occurs in any tube, an insufficient amount of hemolysin has been produced to warrant bleeding of the rabbit. Further injections may be given followed by titrations just described, or the animal may be discarded, and other animals injected.

If hemolysis occurs in the first two tubes only, the hemolysin is too weak to justify the bleeding of the animal.

If, however, hemolysis occurs in any tube higher than Tube 2, the animal should be bled to the point of exsanguination and the final titration made.

The rough titration just described gives an index of the quantity of hemolysin present, so that the final titration may be made with dilutions suggested by the results of the first titration. For example, if hemolysis occurs in the tube containing hemolysin diluted 1 drop to 300 c.c. saline, and partial hemolysis occurs in the tube containing hemolysin diluted 1 drop in 400 c.c. saline, the final dilution lies between 1 drop in 300 c.c. and 1 drop in 400 c.c. The final titration would, in this case, be made with dilutions of 1:280; 1:300; 1:320; 1:340; 1:360; 1:380; 1:400.

If the rough titration of hemolysin warrants the bleeding of the rabbit, the animal is bled to exsanguination[1] and the blood collected in sterile 50 c.c. Rockefeller tubes with corks.

Allow the blood to clot, keeping the tubes slanted.

Loosen the clot by striking firmly against the palm of the hand.

Centrifuge at high speed for fifteen to thirty minutes to separate the serum from the clot.

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[1] See p. 2040.

Decant the supernatant serum into sterile Rockefeller tubes, using all sterile precautions.

Cork the tubes to prevent bacterial contamination.

Inactivate at 56° C. for thirty minutes.

Place the hemolysin in 5 c.c. quantities in sterile ampules, using a sterile syringe. Seal the ampules in the flame.

Leave enough of the hemolysin in a sterile test tube, corked, to allow for the final titration.

Keep the ampules in the refrigerator until ready for use.

After the final dilution of the hemolysin is determined, label all ampules with the dilution just determined, the titer (0.5 c.c.), and date of manufacture.

The final titration of hemolysin is carried out according to Table 190.

The complement must be obtained fresh, and diluted and titrated, using a known hemolysin for the titration. The hemolysin to be titrated is diluted in saline in the quantities given in the preceding paragraph, or other dilutions are made, as the case may be.

TABLE 190.—CHART FOR THE FINAL TITRATION OF HEMOLYSIN IN THE EXAMPLE CITED

TUBES	1	2	3	4	5	6	7
Dilutions of hemolysin	1 drop in 280 c.c. saline	1 drop in 300 c.c. saline	1 drop in 320 c.c. saline	1 drop in 340 c.c. saline	1 drop in 360 c.c. saline	1 drop in 380 c.c. saline	1 drop in 400 c.c. saline
Saline	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Complement*	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Total quantity	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.

*The quantity of complement varies according to a previous titration.

Place in the water-bath at 37°-40° C. for fifteen minutes.

The last tube in which hemolysis occurs is the unit.

The final dilution of hemolysin to be used in the Wassermann reaction will be four times the strength of the hemolysin in the last tube showing hemolysis.

If no tube shows hemolysis, the dilutions are too high, and lower dilutions of the hemolysin will have to be made.

If all the tubes show hemolysis, the dilutions are not high enough, and the titration will have to be carried further.

Example: If the last tube showing complete hemolysis at the end of fifteen minutes' incubation is Tube 4, containing hemolysin diluted 1 drop to 340 c.c. saline, the final dilution of the hemolysin is 1 drop to 85 c.c. saline. After the dilution has been determined, retitrate the same complement used in this titration of hemolysin, using the new hemolysin diluted according to the dilution just determined (in this case 1:85). The titer of the complement should be the same as it was with the old hemolysin. In this manner, each new hemolysin is standardized in its action with complement against the previously titrated hemolysin.

Method of Bleeding Rabbit From the Carotid Artery

Prepare the following sterile instruments:

1 pair sharp pointed small scissors.
1 pair sharp pointed long scissors.
2 seraphine clamps.
2 fine pointed hemostats.
1 pair tissue forceps.
Several pieces of linen thread about 8 inches long.
3 or 4 Rockefeller centrifuge tubes with corks.
Gauze sponges.
2 groove directors, or probes.

Assemble, also, material for anesthetic, iodine, shaving material, rabbit board.

Place the animal on the rabbit board, and give a light anesthetic. Ether is preferable to chloroform.

Shave the hair over the entire front area of the neck.

Pour on a generous quantity of iodine. If too small a quantity of iodine is used, the blood may become contaminated from bacteria on the rabbit's skin. Make a long incision through the skin over the neck. The incision should be at least 4 inches in length, and extend in a lateral line in the center of the neck.

Grasp the probes at the center, and probe down into the muscles until the carotid artery is visible. It will be easily recognized by the arterial beat, and the vagus nerve which is adjacent to it. Do not mistake the jugular vein for the carotid artery.

Lift the artery on the probes, placing the probes sidewise under the artery to lift it.

Separate the artery from the nerve.

Tie off the upper end of the artery with linen thread which has been previously dipped in iodine. Do not tie too tightly, or the arterial wall may be broken. Tie tightly enough so that the artery does not retract through the suture.

Clamp the other end of the artery, about 1½ inches below the thread, with a seraphine clamp.

Cut the thread so that about one-quarter of an inch remains protruding from the double knot.

Pick up one wall of the artery between the thread and the clamp with the hemostat. Be sure not to close the entire artery during this process.

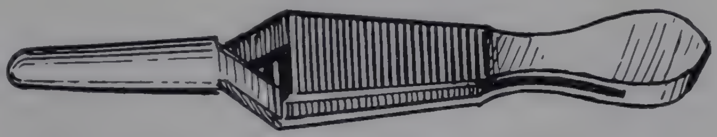

Fig. 595.—Seraphine clamp.

Cut the artery in about the center of the space between the thread and the clamp.

Place a small amount of iodine on the end of the cut artery nearer the clamp. This procedure must not consume too much time.

Direct the end of the artery into a sterile Rockefeller tube, and loosen the seraphine clamp.

The blood spurts through the artery into the tube. Be sure to keep the animal under a light anesthetic during this process. Do not give too heavy an anesthetic.

Allow the blood to run from the artery, filling as many tubes as necessary, until the animal is dying, then give a heavy anesthetic, sacrificing the rabbit.

The bottle is stoppered with a sterile cork immediately after the blood is obtained.

Shipley[1] has devised a board for studies requiring repeated injections into the ear veins of rabbits. It has the advantage of effectively holding rabbits and immobilizing their ears for bleeding or injection procedures.

The significant feature of the apparatus is a mask, shaped to accommodate the anterior three-fourths of the rabbit's head. An inverted, U-shaped collar holds the head firmly in the mask so that the position of the ears is correspondingly stabilized. The head holder is made by molding a liquid thermō-setting plastic[2] around a plaster-of-Paris model which has been shaped to conform to that of the head of a rabbit weighing about 2.5 kilograms. Provisions are made for leaving a hole from the nose to the outside. Within the two horizontal, tubular supports which are included in the plastic casting run two 3/16 inch ball chains 12 inches long. The anterior three-fourths of the rabbit's head is held in the cast by a horse-collar-shaped neckpiece to which are attached the ball chains. Once the rabbit's head is placed in the cast, the operator exerts traction on the chains, and under moderate tension the latter are secured by fitting them into two narrow vertical slots on the ends of the horizontal support tubes.

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[1] Proc. Soc. Exper. Biol. & Med. 63: 75, 1946.

[2] "Catavar 101" (cream colored) or "Catavar 1001" (clear), Catalin Corporation of America, New York, N. Y. Plaster of Paris may also be used.

The body of the rabbit is confined in a 5 by 5 by 12 inch metal box with a hinged lid and clasp. Two flanges fastened to the front of the box prevent the rabbit's legs from reaching the area about the ears. All parts are mounted on a piece of 12 by 27 by ¾ inch plywood, the top and edges of which are covered with sheet metal 0.050 inch thick. For maximum rigidity, durability, and ease in cleaning, stainless steel, chrome-plated brass, and plastic were used. In using this box, by transilluminating the rabbit's ear from below, locating and visualizing particularly the smaller veins is greatly facilitated.

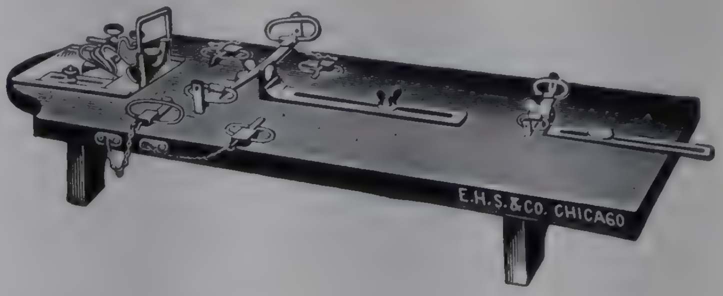

Fig. 596.—Animal board, Latapie. (Courtesy E. H. Sargent & Co.)

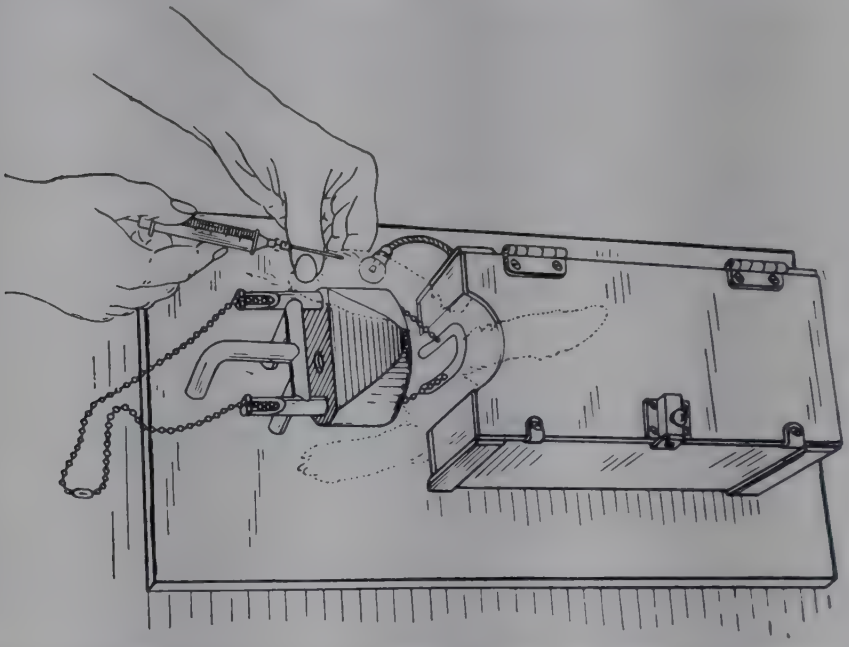

Fig. 597.—The Shipley rabbit board. (Courtesy Dr. R. E. Shipley, Eli Lilly and Company.)

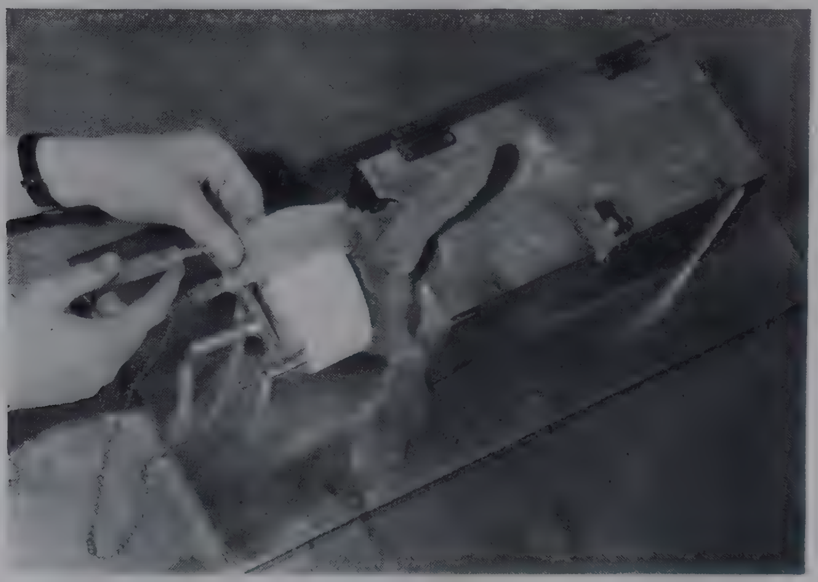

Fig. 598.—Injecting into the marginal ear vein of a rabbit, using the Shipley rabbit board to hold the animal. (Courtesy Dr. R. E. Shipley, Eli Lilly and Company.)

It is rarely necessary to clip or shave the hair over the vein in preparation for injection. A flexible arm with a small socket and bulb attached may be brought into any desired position beneath either ear.

Preparation and Dilution of Sheep Cells for the Wassermann Test

Prepare a 500 c.c. colorless wide-mouth bottle with numerous glass beads. The glass beads are made by breaking up stirring rods to about 1-inch lengths, and rounding the ends in the flame. Cork and sterilize in the dry heat sterilizer at 170° C. for two hours.

Obtain sheep blood from the abattoir, filling the bottle to about two-thirds its capacity. (Sheep may be kept on the premises. In this case, the blood is withdrawn with a syringe from the jugular vein, and defibrinated in the same manner.) Shake vigorously for about fifteen minutes, or until defibrination is complete.

Filter through gauze to remove all particles of fibrin, collecting the filtrate in 50 c.c. sterile Rockefeller tubes with corks. A sterile funnel is used.

Cork tightly.

Place a mark with a wax pencil at the top of the sheep cells.

Centrifuge at high speed for fifteen to thirty minutes to throw down the cells.

Using a sterile transfer pipette (urethral syringe), remove and discard the supernatant fluid.

Fill to the wax pencil mark with sterile physiologic saline.

Recentrifuge.

Decant the supernatant fluid, replacing it with fresh saline, and recentrifuge.

Continue this process until the supernatant fluid is colorless. This is usually accomplished in five or six washings.

After the last centrifuging, remove the clear, colorless supernatant fluid, and replace with fresh saline.

Cork the tubes and keep in the refrigerator.

Fresh sheep cells should be prepared in this manner at least once or twice a week. In summer, the sheep cells will rapidly undergo hemolysis unless unusual care is exercised in keeping them cold.

For use in the Wassermann test, no accurate dilution can be made, since each new quantity of sheep cells may contain more or less cells than the blood previously obtained. It is well, therefore, to dilute to a definite color. About a 3% suspension of the washed cells is used.

Since the diluted sheep cells are used in the titration of complement, no error enters the Wassermann reaction from slight variations in the dilution of the sheep cells. This is part of the hemolytic system of the reaction, and does not take part in the antigen-antibody, or complement fixation, reaction which gives a positive Wassermann test.

Technic of the Wassermann Test

All sera for the Wassermann test are arranged in chronologic order, and the numbers recorded after a letter which represents their place in the day's work. After recording, each blood is prepared in the manner described under **Patient's Serum.** (Page 2032.)

Obtain guinea pig serum, as described above and titrate.

Dilute the hemolysin, antigen, and sheep cells. In diluting the antigen always add the antigen to the saline slowly.

All tests on bloods for the Wassermann reaction are made at the same time. The sera are carried through the day's work with a letter, or a number, or both, according to the routine of the laboratory.

Have on hand known + + + + positive serum and known negative serum.

The water-bath for carrying out the test must be regulated at 37°-40° C. before the tests are made.

The inactivator is set up and regulated at 56° C. before the serum is prepared.

Fresh saline is used in the tests.

In Table 191, the test on only one serum is given. Tubes A and A^1 represent the unknown serum, Tubes Positive and Positive^1 receive known + + + + positive serum,

TABLE 191.—THE WASSERMANN REACTION ON ONE SERUM, AND THE CONTROLS

TUBES	A	A^1	POS.	POS.^{1}	NEG.	NEG.^{1}	X	Y	Z
Patient's serum	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	------	------	------
Saline	0.6 c.c.	0.8 c.c.	0.6 c.c.	0.8 c.c.	0.6 c.c.	0.8 c.c.	0.8 c.c.	1.1 c.c.	1.3 c.c.
Complement	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	------	------
Antigen	0.2 c.c.	------	0.2 c.c.	------	0.2 c.c.	------	0.2 c.c.	0.2 c.c.	------
Place in the water-bath at 37°–40° C. for 30 minutes.									
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Place in the water-bath at 37°–40° C. for 30 minutes.									
Total quantity	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.	2.8 c.c.
Results expected	?	Hem.	No hem.	Hem.	Hem.	Hem.	Hem.	No hem.	No hem.

and Tubes Negative and Negative^1 receive known negative serum. It will be noted that the quantity of patient's serum in these tubes is the same as that used in the test.

A different pipette is used for measuring each new serum.

Since Tube A represents the unknown serum, the result is not anticipated.

Tube A^1 should always show hemolysis, positive shows no hemolysis, etc.

In carrying out the test, all sera are placed in the tubes. Then the saline, then the complement, etc.

The quantity of complement varies according to the previous titration result.

The quantity of saline varies according to the titer of the complement.

In racking up the tubes, place all of the serum control tubes (represented in the chart as A^1, Pos.^{1}, Neg.^{1}) in the back row of the double holed rack. In this way, all serum tubes which do not receive antigen are in the back row.

X, Y, and Z are placed in the front row.

After taking the racks from the water-bath, observe the back row of tubes first, since these are the anticomplementary controls. They should all show hemolysis.

Next, observe all the control tubes, Pos., Neg., X, Y, Z.

If the controls are as they should be, record the results of the Wassermann tests. No hemolysis represents a ++++ positive reaction. Complete hemolysis is a negative reaction. Degrees of positive reaction are judged by the amount of hemolysis; thus if half of the cells are hemolyzed, it is ++, etc.

If the controls give any reaction other than that desired, proceed in the correction of the tests before making reports.

Explanation of Tubes

Tube A represents a Wassermann test on an unknown blood serum. A tube similar to this, together with its serum control, is used for each serum tested. Since it is the patient's blood, the reaction is not known until the racks have been removed from the water-bath the last half hour. If complete hemolysis has occurred, the blood is negative. If complete or partial inhibition of hemolysis has occurred, compare with the result in the test on the known positive and negative sera to judge the degree of the reaction. The report is made on the result in this tube.

Tube A^1 is a test on the serum used in Tube A to determine whether or not it is anticomplementary. Complement is present, but antigen is omitted. No reaction should take place between the patient's serum and the complement unless the serum is anticomplementary. If such is the case, the result in this tube will be complete inhibition of hemolysis, and the report on the result of the Wassermann test of this blood will read: "Test cannot be made, since the serum is anticomplementary." If the serum is not anticomplementary the result in this tube will be complete hemolysis of the sheep cells. A tube corresponding to Tube A^1 is used for each serum introduced into the Wassermann reaction.

Tube Positive is an actual Wassermann test on a known ++++ positive serum. All the reagents are used. This tube, together with Tube Negative, serves as a rough titration of the antigen used in the day's work. The result in this tube checks the reaction of the day's antigen with a positive serum, showing by the result (complete inhibition of hemolysis) that the antigen used is capable of detecting antibodies when they are present in the serum. It, therefore, is a control over a **negative reaction** on an unknown blood. Only one known positive serum is needed to check the antigen each day. If the result in this tube is complete hemolysis of the sheep cells, instead of inhibition of hemolysis, either the serum has deteriorated, or the antigen is not of the correct strength. Before changing the antigen, and making all the tests anew, it is wise to make another positive test using another serum.

Tube Positive¹ is an anticomplementary control over the serum used in Tube Positive. It corresponds (in its relation to Tube Positive) to Tube A¹.

Tube Negative is an actual Wassermann test on a known negative serum. All the reagents are used. This tube, together with Tube Positive, serves as a rough titration of the day's antigen. Since it is a test on a known negative serum, the result should be complete hemolysis, or a negative Wassermann reaction. It is a control over a **positive reaction** on an unknown serum, since it definitely proves that were the serum negative, the reaction between complement, hemolysin, and sheep cells would not be interfered with.

Tube Negative¹ is an anticomplementary control over the serum used in Tube Negative. It corresponds to Tube A¹.

Tube X is a control over the action of antigen with complement. Occasionally an antigen will become anticomplementary. In such case, every tube containing both complement and antigen will show inhibition of hemolysis. Manifestly such a result is incorrect. If an antigen is anticomplementary, a new antigen must be employed in the Wassermann test. This tube should show complete hemolysis, since patient's serum is omitted, and there should be nothing to interfere with the action of complement, hemolysin, and sheep cells.

Tube Y is a control over the action of antigen with hemolysin and sheep cells. It should not cause hemolysis of the sheep cells. The result will, therefore, be inhibition of hemolysis. If hemolysis occurs, every tube containing antigen will likewise show complete hemolysis, and this antigen cannot be used in the Wassermann reaction.

Tube Z is a control over the saline used in the day's work. When the reaction is as it should be, there should be complete inhibition of hemolysis. The saline used should not be so weak as to cause hemolysis of the cells. Too strong a saline solution was guarded against in the titration of complement which was carried out just prior to the making of the Wassermann tests. If the saline solution had been too strong, no titer would have been obtained. The action of hemolysin with complement and sheep cells was likewise checked in the preliminary titration of complement.

If all reagents are as they should be, the results of the Wassermann tests, as shown in Tubes A, B, C, etc., may be reported.

Tubes Positive and Negative are used for comparative purposes in judging the degree of a positive reaction. Complete inhibition of hemolysis with a deposit of sheep cells on the bottom of the tube is ++++. Complete hemolysis of the sheep cells is negative. Any degree of positive reaction between ++++ and borderline must be judged by the worker.

THE WASSERMANN TEST OF SPINAL FLUID

The spinal fluid should be clear and free from blood. If blood is present, obtain a new specimen preferably, or centrifuge the fluid and decant the clear supernatant fluid for the test.

The same reagents are used for this test as for the Wassermann tests on blood serum.

Complement is titrated in the usual manner.

If the spinal fluid is tested along with blood serum, the same set of controls, namely, Tubes Positive, Positive¹, Negative, Negative¹, X, Y, and Z, are used. These tubes represent reagent controls, and consequently one set controls all reagents used either for blood serum or for spinal fluid. If the spinal fluid is tested alone, a set of controls is made. Blood serum is used in Tubes Positive, Positive¹, Negative, Negative¹.

The quantity of complement varies according to the titer.

The amount of saline varies according to the amount of complement used in the test.

TABLE 192

TUBES	1	2	3	4	5	6	CONTROL
Spinal fluid	1.0 c.c.	0.8 c.c.	0.6 c.c.	0.4 c.c.	0.2 c.c.	0.1 c.c.	1.0 c.c.
Saline	0.0 c.c.	0.2 c.c.	0.4 c.c.	0.6 c.c.	0.8 c.c.	0.9 c.c.	0.2 c.c.
Complement	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.	0.3 c.c.
Antigen	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	------
Place in the water-bath at 37°-40° C. for 30 minutes.							
Hemolysin	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Sheep cells	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Place in the water-bath at 37°-40° C. for 30 minutes. **Total in each tube is 3.0 c.c.**							
Results expected	?	?	?	?	?	?	Hemolysis

Tube 7 is placed behind the six "test" tubes in the rack. It is the anticomplementary control. Antigen is omitted. If complete hemolysis of the sheep cells does not occur in this tube, the spinal fluid is anticomplementary, and no Wassermann test can be made.

The tube in which the strongest reaction will occur is Tube 1, in which 1.0 c.c. of spinal fluid is used. In many cases, this is the only tube which will show a positive reaction. This is true especially of treated cases. If the quantity of spinal fluid submitted to the laboratory is small, Tube 1 must always be made. Other tubes may be omitted, since the result in each tube is reported.

Any quantity between 0.2 c.c. and 1.0 c.c. of spinal fluid may be used in the control tube, but if there is sufficient spinal fluid to permit it, use 1.0 c.c. in this tube. If smaller quantities are used, make up the difference with saline.

Report the result in each tube, as follows:

Example:

1.0 c.c.—positive.
0.8 c.c.—positive.
0.6 c.c.—positive.
0.4 c.c.—negative.
0.2 c.c.—negative.
0.1 c.c.—negative.

A negative reaction is complete hemolysis in each tube.

A positive reaction is inhibition of hemolysis in all tubes, or in all but Tube 6, or in all tubes except 6 and 5, etc. If Tube 1 is the only tube showing inhibition of hemolysis, this is a positive reaction, since frequently the quantity of syphilitic substance in spinal fluid is so small that no quantity less than 1.0 c.c. will give a positive reaction.

THE KOLMER COMPLEMENT FIXATION TESTS FOR SYPHILIS[1]

JOHN A. KOLMER, M.D.*

Two methods are commonly used: (1) the qualitative test with serum and spinal fluid; and (2) the quantitative test.

Equipment.—

Pipettes (Fig. 599).—The following pipettes are recommended:

1 c.c. graduated in 0.01 c.c. to tip
5 c.c. graduated in 0.10 c.c.
10 c.c. graduated in 0.10 c.c.

Three 1 c.c. pipettes certified by the Bureau of Standards are recommended for measuring hemolysin, corpuscles, and antigen and a 2 c.c. pipette graduated in 0.10 c.c. for complement.

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*Personal communication, furnished by Dr. John A. Kolmer. Revised according to 1955 Manual, Serologic Tests for Syphilis, U. S. Department of Health, Education, and Welfare, Public Health Publication No. 411.

[1] Kolmer, J. A.: Am. J. Syph. 6: 82, 1922.

Test Tubes.—For the routine quantitative and qualitative tests, which have a total volume of 3.0 c.c., test tubes measuring 8.5 by 1.5 cm. (inside diameter) with rounded bottoms and no lips are employed.

Cylinders.—Glass stoppered, graduated (100 and 500 c.c. capacity) to be used for measuring amounts over 50 c.c.

Test Tube Racks.—Galvanized wire racks carrying 12 rows of 6 tubes each (Fig. 600).

Water Bath.—Any easily regulated water bath is suitable, or a simple galvanized pan carrying water to a depth of 8 centimeters can be used satisfactorily at 55° or 37° C. (Figs. 601 and 602).

Refrigerator.—Any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

Method of Cleaning Glassware.—All glassware should be chemically clean and preferably sterile. To clean tubes and flasks, empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in dry heat sterilizer at about 160° C.

Pipettes should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipettes, rinse thoroughly in running tap water, place in a metal box or wire basket, and sterilize in the oven.

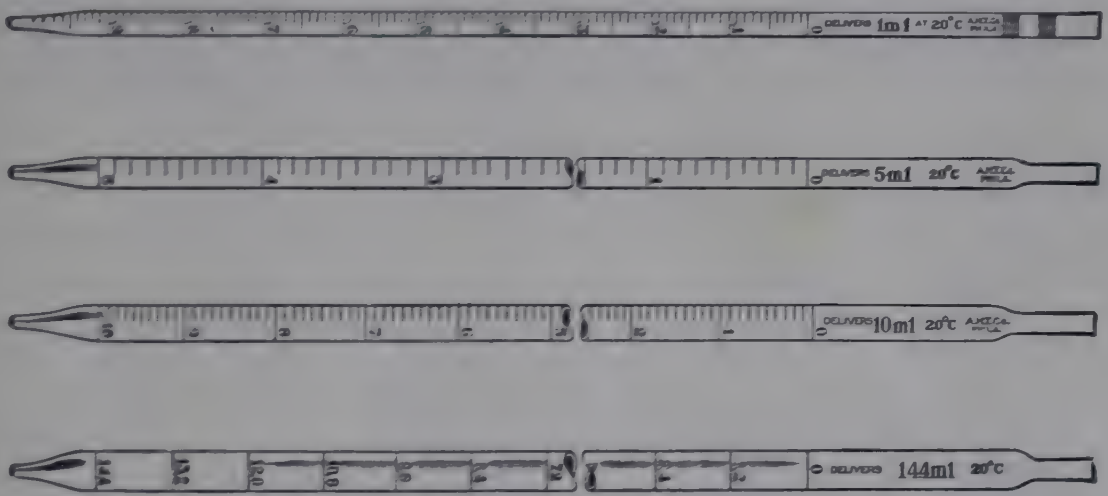

Fig. 599.—Pipettes recommended for the Kolmer complement fixation test.

Flasks should be plugged with cotton and sterilized in the oven for 30 minutes at 160° C.

If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for 24 hours. Rinse thoroughly in running tap water and proceed with the washing as described.

Reagents.—

Saline Solution.—Dissolve 8.5 gm. of dry, C.P., sodium chloride and 0.1 gm. magnesium sulphate in 1,000 c.c. of freshly distilled water. If the salt has absorbed moisture it should be dried in the dry heat oven for 10 or 15 minutes before weighing. Filter solution through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for 1 hour before use (not essential if immediately used).

A satisfactory saline solution should not of itself be hemolytic when 1 or 2 drops of washed corpuscles are added to 5 c.c. of the solution in a test tube followed by water bath incubation for 1 hour. Neither should it be antihemolytic as determined by the hemolysin or complement titrations. The antisheep hemolysin should give a unit of at least 0.5 c.c. of a 1:4,000 dilution when titrated with 0.3 c.c. of 1:30 complement and 0.5 c.c. of a 2 per cent suspension of washed sheep corpuscles with a water bath incubation of 1 hour. It is possible that failure of hemolysis may be due to defective hemolysin or complement or to the use of corpuscles of increased resistance to serum hemolysis.

When unusually low complement titers are obtained, Kolmer recommends adding 40 mg. $CaCl_2 \cdot 2H_2O$ to each liter of saline. When this is done, the complement should not be used at a dilution higher than 1:37 (for 2 full units).

Preparation of Sheep Corpuscles (Indicator Antigen).—Sheep blood may be obtained at an abattoir or by bleeding a sheep from the external jugular vein. In a clean (but not necessarily sterile) quart-sized Mason jar, place 30 c.c. of a 10 per cent solution of sodium citrate in saline solution and 2 c.c. of Formalin. At the abattoir have the jar almost filled with *fresh* blood (blood kept over in buckets is unsatisfactory), screw on the top, mix well with the citrate-Formalin solution, and keep in a refrigerator. Ordinarily it is fit for use over a period of 2 to 3 weeks at least, but as soon as the corpuscles become too fragile a fresh supply should be secured. Boerner and Lukens advise keeping blood for 48 hours in the refrigerator before using.

If preservation is not desired, glass beads alone may be placed in the jar, and after filling with blood, it should be thoroughly shaken for defibrination. Blood collected in this manner will keep at a low temperature (jar placed on a block of ice) for about a week.

Filter a small quantity of blood through cotton into a *graduated* centrifuge tube (Fig. 603). Allow twice as much blood as the amount of cells required. Add 2 or 3 volumes of saline solution. Centrifuge at a moderate velocity until it is ascertained that all the corpuscles are thrown down.

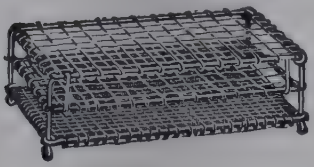

Fig. 600.

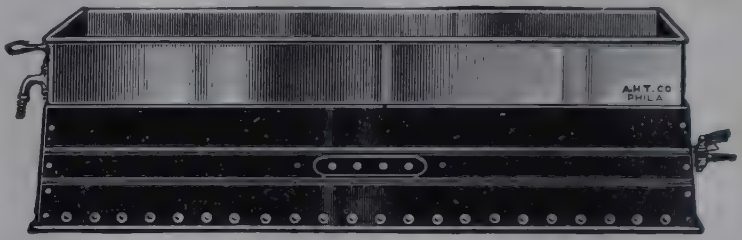

Fig. 601.

Fig. 600.—Test tube rack for use in the Kolmer complement fixation test.
Fig. 601.—Water bath for use in the Kolmer complement fixation test.

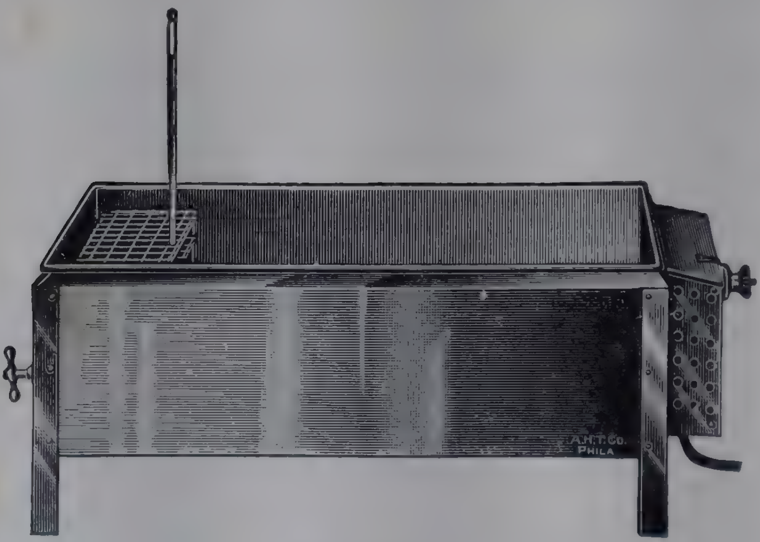

Fig. 602.

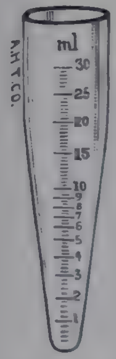

Fig. 603.

Fig. 602.—Water bath for use in the Kolmer complement fixation test.
Fig. 603.—Graduated centrifuge tube used to wash sheep corpuscles.

Remove the supernatant fluid with a capillary pipette or by suction. Add 3 or 4 volumes of saline solution; mix by inverting and centrifuge again for the same length of time.

Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly.

Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. (If more than four washings are necessary, the cells are too fragile for use.)

Read the volume of cells in the centrifuge tube, carefully remove the supernatant fluid, and prepare a 2 per cent suspension by washing the corpuscles into a flask with 49 volumes of saline solution or dilute 2 c.c. of corpuscles with 98 c.c. of saline solution. *Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.*

Preparation of Antisheep Hemolysin.—Give a rabbit 5 or 6 intravenous injections of 5 c.c. of a 10 per cent suspension of washed sheep corpuscles every 5 days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration gives a unit of 0.5 c.c. of 1:4,000 or higher. Separate the serum (it need not be inactivated) and preserve with an equal part of best grade neutral glycerin. Keep in a refrigerator.

Preparation of Complement.—The pooled sera of at least 3 healthy male guinea pigs should be used. Select large, well-nourished animals that have not been fed for 12 hours; avoid pregnant animals.

Anesthetize the pig lightly with ether. Collect the blood from the heart (page 2033) in a sterile test tube, and allow to clot. Loosen the clot with a sterile applicator and centrifuge. Remove the clear supernatant serum and place in a clean, dry, sterile test tube. *Keep in the refrigerator when not in use.*

If but a small amount of serum is required, 4 or 5 c.c. of blood may be obtained from each pig by bleeding from the heart with a 5 c.c. syringe fitted with a short needle, gauge 20.

Complement serum may be preserved for several weeks by adding 0.3 gm. of c. p. sodium chloride to each c.c. of serum. Keep in a dark glass bottle at or near the *freezing point.* To prepare for use, dilute 1 c.c. of serum with 29 c.c. of distilled water. This gives a 1:30 dilution in 1 per cent salt solution. Complement may also be preserved for at least 2 weeks by adding and dissolving 1 gm. of chemically pure sodium acetate in each 10 c.c. of serum (Rhamy). *Sonnenschein's solution* is also recommended:

Sodium acetate (c.p.)	12.0 gm.
Boric acid	4.0 gm.
Sterile distilled water	100.0 c.c.

Equal parts of serum and solution are mixed giving a 1:2 dilution of complement from which a 1:30 dilution is prepared by diluting 1 c.c. with 14 c.c. of saline. By whatever of these chemical methods complement is preserved, it is important to keep the serum frozen or at a temperature not exceeding 2° C. As preserved complement loses first in fixability by syphilis antigen and antibody, it should not be kept for more than 3 or 4 weeks.

Lyophil Complement.[2]—The most satisfactory method for preserving complement is by evaporation in the frozen state in vacuo by the method of Flosdorf and Mudd.[3] It retains both hemolytic activity and fixability for 8 to 12 months and has proved satisfactory. It has the great advantage of being prepared of the pooled sera of a large number of guinea pigs with uniform hemolytic activity and fixability by antigen and antibody. It may be obtained from Sharp and Dohme, Philadelphia, and is dispensed in vacules containing the equivalent of 5 c.c. of fresh serum. These should be kept in a refrigerator until used. By adding 5 c.c. of distilled water the material goes into immediate solution and is ready for use in the same manner as fresh serum. Preservation by the *cryochem process* is also recommended and is more economical.

Preparation of Antigen.—The antigen for the Kolmer test is an alcoholic solution containing 0.03 per cent cardiolipin, 0.05 per cent lecithin, and 0.3 per cent cholesterol. Each new lot must be tested along with a standard antigen before use. It is best to purchase antigens which have been standardized.* Kolmer[4] states that such antigens are preferred as a mixture of maximum sensitivity consistent with specificity in tests with sera and spinal fluids.

Preparation of Antigen Dilution.—Place the required amount of saline in a flask and add the antigen drop by drop while continuously shaking the flask. Calculate the amount needed for the day's work.

The test dose constitutes 0.5 c.c. of the antigen dilution indicated on the label of the bottle, which is usually 1:150.

Keep the antigen dilution at room temperature in a stoppered flask.

Allow to stand at room temperature for at least 1 hour before use.

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*Kolmer cardiolipin antigen may be purchased from Sylvana Chemical Company, Orange, N. J.

[2] Kolmer, J. A., Richter, C. E., and Lynch, E. R.: Am. J. Clin. Path. 8: 522, 1938.

[3] J. Immunol. 29: 389, 1935.

[4] Kolmer, J. A., and Lynch, E. R.: J. Ven. Dis. Inform. 29: 166, 1948.

Preparation of Sera.—All specimens are lined up and properly labeled.

The sera are removed from the clots with capillary pipettes to test tubes properly labeled. Great care is required to prevent errors in labeling and confusion of sera. Each serum should be free of corpuscles, otherwise it is necessary to break up the clots with wooden applicators (one for each serum) and centrifuging for clear serum. Slight tinging with hemoglobin does no harm. Sera containing large amounts of hemoglobin are likely to be anticomplementary and unsatisfactory for both complement fixation and precipitation tests.

Removal of Natural Antisheep Hemolysin From Sera.—

It is not necessary to remove the natural antisheep hemolysins by absorption with *thoroughly washed sheep corpuscles* although this tends to increase the sensitiveness of complement fixation reactions and especially in the case of sera containing small amounts of syphilis antibody. Kolmer recommends the routine removal of natural hemolysins when conditions permit in order to secure reactions of maximum sensitiveness, but when large numbers of sera require testing it may be omitted.

To each is added *a drop of washed sheep corpuscle sediment for approximately each 2 c.c. of blood and serum* as gauged by inspection.

Each specimen is then thoroughly mixed with a wooden applicator (one for each serum).

All are then placed in a *refrigerator for 15 minutes* to enable the sheep corpuscles to absorb the hemolysins with none or but a minimum and harmless amount of hemolysis.

All specimens are now centrifuged and the sera separated into test tubes properly labeled.

The tubes of plain or absorbed sera are now placed in a water bath at 55° C. for *15 to 20 minutes,* when they are ready for testing.

Preparation of Spinal Fluids.—*These are usually tested without any preliminary preparation* as they do not contain enough natural antisheep hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged. Otherwise no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more than 3 days old, in which case they may be heated at 55° C. for 15 minutes to remove thermolabile anticomplementary substances. As a general rule, however, spinal fluids are not anticomplementary unless heavily contaminated with bacteria (cloudy). If a large amount of blood is present, the spinal fluid cannot be used.

The Regular Quantitative Kolmer Complement Fixation Test

TITRATION OF HEMOLYSIN

It is advisable to make this titration each time the complement fixation test is conducted.

Prepare a **stock dilution** of 1:100 hemolysin:

Saline solution	94.0 c.c.
Phenol, 5% in saline solution	4.0 c.c.
Glycerinized hemolysin (50%)	2.0 c.c.

Mix the phenol and saline solution well before adding the glycerinized hemolysin. This may be kept in a refrigerator for several months, unless a precipitate develops, in which case it must be discarded.

For titration: Dilute to 1:1,000 by adding 0.5 c.c. of the 1:100 dilution to 4.5 c.c. saline solution.

Prepare higher dilutions in a series of 10 tubes as in Table 193.

TABLE 193

TUBE	0.5 C.C. HEMOLYSIN: DILUTION:	C.C. SALINE SOLUTION	DIRECTIONS	FINAL DILUTION OF HEMOLYSIN
1	1:1,000	—	—	1:1,000
2	1:1,000	0.5	Discard 0.5 c.c.	1:2,000
3	1:1,000	1.0	Transfer 0.5 c.c. to tube 6. Discard 0.5 c.c.	1:3,000
4	1:1,000	1.5	Transfer 0.5 c.c. to tube 7. Discard 1 c.c.	1:4,000
5	1:1,000	2.0	Transfer 0.5 c.c. to tube 8. Discard 1.5 c.c.	1:5,000
6	1:3,000	0.5	Transfer 0.5 c.c. to tube 9	1:6,000
7	1:4,000	0.5	Transfer 0.5 c.c. to tube 10	1:8,000
8	1:5,000	0.5	Discard 0.5 c.c.	1:10,000
9	1:6,000	0.5	Discard 0.5 c.c.	1:12,000
10	1:8,000	0.5	Discard 0.5 c.c.	1:16,000

Mix the contents of each tube thoroughly.

Carry out the titration in these tubes, each containing 0.5 c.c. of diluted hemolysin.

Prepare a 1:30 dilution of complement for hemolysin and complement titration by diluting 0.2 c.c. of complement serum with 5.8 c.c. saline solution.

Prepare a 2 per cent suspension of sheep corpuscles.

In the series of 10 tubes, Table 193, add to all tubes:

0.3 c.c. of 1:30 complement
1.7 c.c. saline solution
0.5 c.c. sheep corpuscles.

Mix the contents of each tube and incubate in the water bath at 37° C. for 1 hour. Read the unit of hemolysin. The *unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the complement and antigen titrations and in the complement fixation tests. Hemolysin is so diluted that 0.5 c.c. contains 2 units. For example, if the unit equals 0.5 c.c. of 1:6,000, two units equal 0.5 c.c. of 1:3,000. Dilute just enough hemolysin for the complement titration and the complement fixation tests. *Keep hemolysin and corpuscles in suspension in the refrigerator when not in use.*

Table 194 shows how the dilutions are made so that 0.5 c.c. carries two units.

TABLE 194

1 UNIT 0.5 C.C. OF—	2 UNITS WOULD BE 0.5 C.C. OF—	PREPARED BY DILUTING 1 C.C. OF STOCK 1:100 WITH FOLLOWING AMOUNTS OF SALINE C.C.
1: 1,000	1: 500	4
1: 2,000	1:1,000	9
1: 3,000	1:1,500	14
1: 4,000	1:2,000	19
1: 5,000	1:2,500	24
1: 6,000	1:3,000	29
1: 8,000	1:4,000	39
1:10,000	1:5,000	49

High titer hemolysin is recommended and the unit should be 0.5 c.c. of 1:4,000 or higher.

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and two units are added to all tubes of the complement titration, etc.

TITRATION OF COMPLEMENT

For the complement titration use 1:30 dilution of complement prepared above. *Dilute antigen so that the dose employed in the main tests is contained in 0.5 c.c.* This dilution is made by placing the required amount of saline solution in a flask and adding antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough antigen dilution for the complement titration and the complement fixation tests.

In a series of 8 test tubes set up, the complement titration is as in Table 195.

TABLE 195

TUBE	COMPLEMENT, C.C. (1:30)	ANTIGEN DOSE, C.C.	SALINE SOLUTION, C.C.		HEMOLYSIN C.C. (2 UNITS)	CORPUSCLES, C.C. (2 PER CENT)	
1	0.2	0.5	1.3	Water bath 37° C. for 1 hour	0.5	0.5	Water bath 37° C. for 1 hour
2	0.25	0.5	1.3		0.5	0.5	
3	0.3	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2		0.5	0.5	
5	0.4	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1		0.5	0.5	
7	0.5	0.5	1.0		0.5	0.5	
8	None	None	2.5		None	0.5	

The smallest amount of complement just giving complete sparkling hemolysis is the *exact unit.* The next higher tube is the *full unit* which contains 0.05 c.c. more complement. In conducting the antigen titration and complement fixation tests, *two full units* are employed and so diluted as to be contained in 1 c.c. as per the following example:

Exact unit:	0.3 c.c.
Full unit:	0.35 c.c.
Dose (two full units):	0.7 c.c.

To calculate the dilution to employ so that 1 c.c. contains the dose of two full units, divide 30 by the dose: $\frac{30}{0.7} = 43$ or 1 c.c. of 1:43 dilution of serum.

Table 196 gives additional examples:

TABLE 196

EXACT UNIT, C.C.	FULL UNIT, C.C.	TWO FULL UNITS, C.C.	DILUTION TO USE	PREPARATION
0.2	0.25	0.5	1:60	1 c.c. serum + 59 c.c. saline
0.25	0.3	0.6	1:50	1 c.c. serum + 49 c.c. saline
0.3	0.35	0.7	1:43	1 c.c. serum + 42 c.c. saline
0.35	0.4	0.8	1:37	1 c.c. serum + 36 c.c. saline
0.4	0.45	0.9	1:33	1 c.c. serum + 32 c.c. saline
0.45	0.5	1.0	1:30	1 c.c. serum + 29 c.c. saline
0.5	0.55	1.1	1:27	1 c.c. serum + 26 c.c. saline

It is always advisable to dilute complement serum with cold saline solution instead of with saline kept at room temperature. Undiluted and especially diluted complement serum should always be kept in a refrigerator when not in use. Exposure of diluted complement to room temperature for over an hour may result in some deterioration.

Occasionally hyperactive complement yields a unit of 0.2 to 0.25 c.c. of 1:30, but when this occurs it is necessary to take arbitrarily 0.3 c.c. as the exact unit as less complement falls below the absolute minimum and is likely to be unsatisfactory.

KOLMER QUANTITATIVE TEST ON SERUM AND SPINAL FLUID

(A) Serum:

Place 8 tubes for each serum in a rack.

Include 3 tubes for reagent controls and several tubes for graded known positive sera and 1 tube for negative serum for the day's work.

For each serum add saline in the following amounts:

0.9 c.c. into tube 1 and 0.5 c.c. saline in tubes 2 through 8.

Place the indicated amounts of saline in each reagent control as follows:

Antigen control	0.5 c.c.
Hemolytic control	1.0 c.c.
Corpuscle control	2.5 c.c.

Place 0.6 c.c. inactivated serum in tube 1.

Mix and transfer 0.5 c.c. to tube 8 (serum control) and to tube 2.

Mix the contents of tube 2 and transfer 0.5 c.c. to tube 3.

Continue in this manner through tube 7. Mix the contents of tube 7 and discard 0.5 c.c.

The tubes now contain 0.5 c.c. serum diluted, respectively, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and no serum.

Add 0.5 c.c. diluted antigen to the first 7 tubes of each serum test, and to the antigen control.

Shake the racks to mix thoroughly.

Allow to stand at room temperature for 10 to 30 minutes.

Prepare the diluted complement during this interval.

Add 1 c.c. of diluted complement, containing 2 full units, to each tube of the serum tests and to the control serum tests.

Add 1 c.c. diluted complement to the antigen control and the hemolytic system control.

Mix by shaking the racks well, and place in a refrigerator at 6° to 8° C. for 15 to 18 hours.

Prepare the volume of diluted hemolysin needed for the test, allowing 0.5 c.c. containing 2 units for each tube. Prepare a slight excess.

Remove the racks from the refrigerator and place immediately in a water bath at 37° C. for 10 minutes.

Remove from the water bath and add 0.5 c.c. of the diluted hemolysin to all tubes of the test except the corpuscle control.

Add 0.5 c.c. of 2% sheep red cell suspension, prepared the previous day, to all tubes. Agitate the cell suspension occasionally to insure even suspension of cells while adding to the tests.

Shake the rack thoroughly and return to the water bath at 37° C.

Examine the controls at 5-minute intervals. The period of secondary incubation will be determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control sera.

The reading time should be at least 10 minutes more than that required to hemolyze the antigen and hemolytic system controls, but must not exceed a total of 60 minutes.

When control sera are not available, the secondary incubation period should be terminated 10 minutes after the hemolytic system control and the antigen control hemolyze. This period must never exceed 1 hour.

Record hemolysis and degrees of hemolysis as indicated in Tables 197 to 199.

The serum control must show complete hemolysis.

Quantitative tests may be reported in terms of the highest dilution giving a reactive result. If a reactive result is obtained with a 1:64 dilution of the serum, higher dilutions may be made and tested.

TABLE 197.—REPORTING KOLMER QUALITATIVE TESTS

TEST READING	CONTROL READING	REPORT	TEST READING	CONTROL READING	REPORT
4+	–	Reactive	3+	3+	Anticomplementary
3+	–	Reactive	3+	2+	Anticomplementary
2+	–	Reactive	3+	1+	Weakly reactive
1+	–	Reactive	3+	±	Reactive
±	–	Weakly reactive	2+	2+	Nonreactive
–	–	Nonreactive	2+	1+	Nonreactive
4+	4+	Anticomplementary	2+	±	Weakly reactive
4+	3+	Anticomplementary	1+	1+	Nonreactive
4+	2+	Weakly reactive	±	±	Nonreactive
4+	1+	Reactive			

TABLE 198.—REPORTING KOLMER QUANTITATIVE RESULTS IN TERMS OF THE HIGHEST DILUTION GIVING A REACTIVE RESULT (SERUM OR SPINAL FLUID)

1:1	1:2	1:4	1:8	1:16	1:32	1:64	REPORT
4	3	1	–	–	–	–	Reactive, 1:4 dilution, or 4 dils.
4	–	–	–	–	–	–	Reactive (4+), undiluted, or 1 dil.
4	4	3	2	–	–	–	Reactive, 1:8 dilution, or 8 dils.
4	4	4	4	4	1	–	Reactive, 1:32 dilution, or 32 dils.
3	1	–	–	–	–	–	Reactive, 1:2 dilution, or 2 dils.
1	–	–	–	–	–	–	Reactive (1+), undiluted, or 1 dil.
2	1	±	–	–	–	–	Reactive, 1:2 dilution, or 2 dils.
±	–	–	–	–	–	–	Weakly reactive
–	–	–	–	–	–	–	Nonreactive
4	4	4	4	4	4	4	Proceed with further dilutions and tests

TABLE 199.—KOLMER QUANTITATIVE TEST REPORTING (AFTER 1 HOUR SECONDARY INCUBATION AT 37° C.)

TEST READING	CONTROL READING	REPORT
4 4 4 4 4 2 –	4+	Anticomplementary
4 4 3 – – – –	2+	Reactive
4 4 1 – – – –	1+	Reactive
4 4 1 + – – –	±	Reactive
3 2 – – – – –	±	Reactive
4 4 1 – – – –	3+	Weakly reactive
3 2 – – – – –	1+	Weakly reactive
2 1 – – – – –	±	Weakly reactive
3 – – – – – –	±	Reactive
1 – – – – – –	±	Nonreactive
1 – – – – – –	1+	Nonreactive
2 – – – – – –	1+	Nonreactive
2 – – – – – –	2+	Nonreactive

(B) Spinal Fluid:

Use 6 tubes for each spinal fluid. If these tests are made at the same time as tests on the sera are made, no further controls need be made. If made alone, include the controls given above.

Place 0.5 c.c. of saline in tubes 2, 3, 4, 5, 6.

Place 0.5 c.c. spinal fluid in tube 1, and 0.5 c.c. in tubes 2 and 6.

Mix the contents of tube 2 and transfer 0.5 c.c. to tube 3, and continue in this manner through tube 5. Discard 0.5 c.c. from tube 6. The dilutions of spinal fluid in their respective tubes is now 1:1, 1:2, 1:4, 1:8, and 1:16. Tube 6 is the control, and contains 0.5 c.c. undiluted spinal fluid.

Add 0.5 c.c. of diluted antigen to the first 5 tubes of each spinal fluid test.

Shake the racks.

Allow to stand at room temperature for 10 to 30 minutes.

Continue with the test from this point, as in the Kolmer quantitative test for serum, pages 2052 ff.

Read as directed for the Kolmer quantitative test of serum, Tables 197, 198, and 199.

KOLMER QUALITATIVE TEST ON SERUM AND SPINAL FLUID

Set up tubes in a rack, 2 tubes for each serum, and 2 tubes for each spinal fluid. Use, also, control sera of graded reactivity and negatively reacting sera, 2 tubes for each. Set up 4 additional tubes for reagent controls.

Arrange the tubes for the sera and sera controls in two rows.

Place 0.5 c.c. saline into each tube of the second row.

Add saline to the reagent controls as follows:

Antigen control for serum tests	0.2 c.c.
Antigen control for spinal fluid tests	0.5 c.c.
Hemolytic system control	1.0 c.c.
Corpuscle control	2.5 c.c.

Place 0.2 c.c. of each serum in both their tubes, 1 and 2 (front and back rows).

Add 0.5 c.c. spinal fluid to its tubes 1 and 2 (front and back rows).

Add 0.2 c.c. of each control serum to its tubes 1 and 2.

Add 0.5 c.c. antigen dilution to the front tubes of each test (tube 1), and to the 2 antigen control tubes.

Allow to stand at room temperature for 10 to 30 minutes.

Prepare the diluted complement during this interval. The amount needed is equivalent to 1 c.c. for each tube of the test plus a slight excess.

Add 1 c.c. diluted complement containing 2 full units to all tubes of the serum, control serum, and spinal fluid tests, including the antigen control and the hemolytic system control.

Shake the racks well and place in a refrigerator at 6° to 8° C. for 15 to 18 hours.

Prepare the diluted hemolysin needed for the test, allowing 0.5 c.c. containing 2 units for each tube. Prepare a slight excess.

Remove the racks from the refrigerator at regular intervals and place immediately in a water bath at 37° C. for 10 minutes. The interval is determined by the time necessary to add the hemolysin and sheep cell suspension to each rack.

Remove from the water bath and add 0.5 c.c. of diluted hemolysin to all tubes except the corpuscle control.

Add 0.5 c.c. of 2% sheep red cell suspension, prepared the previous day, to all tubes. Agitate the cell suspension occasionally to insure even suspension of the cells during the period when they are being added to the test.

Shake the racks thoroughly and return to the 37° C. water bath for secondary incubation. Examine the controls at 5-minute intervals. The period of secondary incubation is determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control sera. The reading time must be at least 10 minutes longer than that required to hemolyze the antigen and hemolytic system controls, but must not exceed 60 minutes. If control sera are not available, terminate the secondary incubation period 10 minutes after the hemolytic system control and the antigen control hemolyze. This secondary incubation period must not exceed 1 hour.

Read results by comparison with the reading standards and report.

PREPARATION OF READING STANDARDS

Heat tubes of hemoglobin solution, saved from the titration or obtained from control tubes of the day's tests, in a water bath at 56° C. for 5 minutes.

Prepare a 1:6 dilution of 2% sheep corpuscle suspension by adding 5 c.c. of saline solution to 1 c.c. of the 2% suspension.

Prepare reading standards by mixing hemoglobin solution and cell suspension as in Table 200.

TABLE 200

1:6 CORPUSCLE SUSPENSION C.C.	HEMOGLOBIN SOLUTION C.C.	EQUIVALENT FIXATION PER CENT	COMPLEMENT REPORT
3.0	0.0	100	4+
1.5	1.5	50	3+
0.75	2.25	25	2+
0.3	2.7	10	1+
0.15	2.85	5	±
---	3.0	0	–

READING AND REPORTING RESULTS

All serum and spinal fluid controls should show complete hemolysis.

Estimate the individual tube readings by comparison with the reading standards at the end of the secondary incubation period, and record the degree of complement fixation noted, except when the specimen shows inhibition of hemolysis in the control tube.

Read the tubes that have been returned to the 37° C. bath for a full hour's secondary incubation, estimating and recording the degree of complement fixation of each tube and control tube by comparison with the reading standards. See Table 197, page 2053.

Preparation of Anticomplementary Sera for Kolmer Complement Fixation Tests (Modified Sachs Method)

Heat 0.5 c.c. of serum at 55° to 56° C. in a water bath for 15 minutes. If the serum has been previously heated and is being re-tested, heat for 5 minutes.

Add 4.1 c.c. of accurately titrated N/300 hydrochloric acid and mix thoroughly.

After standing ½ hour at room temperature, centrifuge for 10 minutes and discard the sediment.

To the supernatant fluid add 0.4 c.c. of 10% sodium chloride solution. The acid is fixed by the precipitate of globulin; hence neutralization is unnecessary.

This gives a 1:10 dilution of original serum ready for testing as follows:

Arrange 2 rows of 5 test tubes each, and number them 1 to 5. The second row contains the serum controls.

Place 0.5 c.c. of saline in tubes 3, 4, and 5 of both rows.

Add to both rows:

1 c.c. of treated serum to tube 1.

0.5 c.c. of treated serum to tubes 2 and 3.

Mix tube 3 and transfer 0.5 c.c. to tube 4.

Mix tube 4 and transfer 0.5 c.c. to tube 5.

Mix tube 5 and discard 0.5 c.c.

The 5 tubes of each row contain 0.1, 0.05, 0.025, 0.012, and 0.006 c.c. of serum, respectively.

Add 0.5 c.c. of antigen dilution to each tube of the first row.

Add 0.5 c.c. of saline to each tube of the second row.

Shake the rack and allow to stand at room temperature for 10 to 30 minutes.

Add 1 c.c. of diluted complement to all tubes of both rows.

Shake the rack and place in a refrigerator for 15 to 18 hours at 6° to 8° C.

Continue the test as described under the Kolmer Qualitative Tests with Serum and Spinal Fluid, pages 2054 ff.

Record the degree of hemolysis in both the test and the control tubes. All the tubes of the rear row may show complete hemolysis; however, the first and second tubes of the rear row may show slight inhibition of hemolysis.

With nonreactive sera, the corresponding front tubes show the same degree of inhibition of hemolysis, and if the degree is slight, a nonreactive report may be rendered. With reactive sera, inhibition of hemolysis is much more marked in tubes of the front row. Report results as reactive, weakly reactive, and nonreactive.

RETESTING ANTICOMPLEMENTARY SPINAL FLUIDS

Heat the spinal fluid for 15 minutes at 56° C.

Arrange 2 rows of 5 tubes each and number 1 to 5.

Add 0.5 c.c. of saline to tubes 2, 3, 4, and 5 of both rows.

To both rows:

Add 0.5 c.c. of spinal fluid to tubes 1 and 2.
Mix tube 2 and transfer 0.5 c.c. to tube 3.
Mix tube 3 and transfer 0.5 c.c. to tube 4.
Mix tube 4 and transfer 0.5 c.c. to tube 5.
Mix tube 5 and discard 0.5 c.c.

Add 0.5 c.c. of antigen dilution to each tube of the first row.

Add 0.5 c.c. of saline to each tube of the second row.

Shake the rack and allow to stand at room temperature for 10 to 30 minutes.

Add 1 c.c. of diluted complement to all tubes of both rows. Shake the racks and place in a refrigerator for 15 to 18 hours at 6° to 8° C.

Complete the tests as described under Kolmer Qualitative Tests with Serum and Spinal Fluid, pages 2054 ff.

Interpret the results as follows:

FIRST ROW	SECOND ROW	REPORT
4 4 4 1 –	4 1 – – –	Reactive
4 3 2 – –	4 1 – – –	Reactive
4 1 – – –	1 – – – –	Reactive
4 4 2 – –	4 2 1 – –	Nonreactive
3 2 – – –	3 1 – – –	Nonreactive

Analysis of Difficulties

Due to Complement.—When difficulties are observed, the trouble is usually first thought to be due to defective hemolysin or antigen but, since both of these keep very well, they are seldom responsible. In the great majority of instances, difficulties are due to the complement because too low in hemolytic activity or too sensitive to what may be called the occult anticomplementary effects of antigen or serum and particularly a summation of both. This is especially likely to occur during the hot months of the year and likewise when the sera of underweight or pregnant guinea pigs are used as well as those of guinea pigs previously employed for various inoculation tests.* When the unit is higher than 0.5 c.c. of 1:30 dilution, the complement should not be used.

Due to Hemolysin.—As previously stated, this is usually first suspected but probably least likely to be a cause of trouble, especially if the hemolysin has been previously used with success. The unit of antisheep hemolysin should be at least 0.5 c.c. of 1:4,000 and serum of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible even when shipped over long distances.

Due to Corpuscles.—When blood is obtained from an abattoir one is almost sure sooner or later to encounter the corpuscles of occasional animals possessing *increased resistance* to serum hemolysis. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood.

Due to Antigen.—Providing no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system, especially the complement.

Due to Anticomplementary Sera and Spinal Fluids.—Sera and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the controls. After experience has been gained some of these reactions may be safely read, as previously stated, but as a general rule it is safer and wiser to repeat the tests with fresh serum or spinal fluid, especially in the case of those technicians lacking experience in complement fixation work. It is infinitely better to repeat the tests than to run the slightest chance of error, especially the regrettable and almost unpardonable error of rendering a falsely positive report. Sometimes the majority of sera and spinal fluids of a day's work show incomplete hemolysis of the controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests may have to be repeated and for this reason the *unused portions of all sera and spinal fluids should be routinely kept in a refrigerator until the tests are completed in case repetitions are required.* In case difficulties are due to the presence of thermostable anticomplementary substances in sera, they can usually be satisfactorily tested after preparation by the modified method of Sachs.

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*Use only male pigs.

THE HECHT-GRADWOHL COMPLEMENT FIXATION TEST FOR SYPHILIS

The method of performing the Wassermann test described on pages 2031 ff. entailed the use of ''inactivated'' serum, complement derived from a guinea pig, and specific hemolysin developed as a result of repeated injections of washed sheep cells into rabbits. In the years following the original announcement of Wassermann, Neisser, and Bruck of complement fixation as a method of diagnosing syphilis, it was found that many cases of syphilis failed to give a positive Wassermann reaction. It was believed that this failure to obtain a positive Wassermann reaction was due to errors inherent in the performance of the test. Noguchi early determined that the syphilitic substance, or reagin, which gives rise to the positive Wassermann, was partly thermolabile and partly thermostable. In other words, a part of the syphilitic reagin resisted the temperature of 56° C., to which blood serum was subjected in inactivation, and some of it did not. He also showed that in certain syphilitic individuals the thermolabile factor might constitute the larger percentage of the syphilitic antibody. Therefore, in those cases, inactivation would completely render the following Wassermann test negative.

For this reason, efforts were made to utilize active serum so as to keep both the thermolabile and thermostable factors inviolate in the test. The principal methods using active serum that have appeared in the literature are those of M. Stern,[1] Hecht,[2] Gradwohl,[3] Laredde,[4] Levaditi and Latipie, Famulener and Hewitt, McIntyre, Worth and McIntyre, Fernandez, Perilmann, Boas, Mutermilch and Latapie, Plaut, Müller and Lansteiner,[5] and Noguchi.[6]

I began my serological work in 1907 in Wassermann's laboratory, and soon discovered that many cases of syphilis were not detected by the original Wassermann test. I therefore interested myself in the use of active sera for complement fixation and, after trying out a number of methods worked out a modification of the original tests advocated by Hecht, Weinberg, and Laredde. This work, which was begun in 1910, was published in 1914. A number of reports on this work were made and can be found in the literature.[7] Briefly, the method employs blood serum which is not inactivated, and utilizes the native complement and native antisheep hemolysin present in practically all human bloods. Fullest advantage is taken of the fact that the thermolabile and thermostable factors of blood serum are preserved in this method. From 1913 until the present time, that is, over a period of forty years,

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[1] Ztschr. f. Immunitätsforsch. 22: 115, 1914.

[2] Dermat. Wchnschr. 68: 289, 1919.

[3] J. A. M. A. 63: 240, 1914. J. A. M. A. 68: 514, 1917. Am. J. Syph. 1: 450, 1917.

[4] Bull. de derm. 1922, p. 321.

[5] Wien. klin. Wchnschr. 1917, No. 10.

[6] J. Exper. Med. 28: 297, 1918.

[7] Gradwohl, R. B. H.: J. A. M. A. 63: 240, 1914; Lancet-Clinic 112: 624, 1914.

I have used the test that I originated as a routine method, together with the Wassermann test as standardized by Kolmer, and the Kahn precipitation test, later to be described. As a result of an experience with over 100,000 blood sera, I firmly believe that this method affords the most reliable and satisfactory measure for the estimation of the presence of syphilitic reagin in a given blood serum. It presents no difficulties in technical performance nor does it give false positives. It might be added that this work has been confirmed in a number of other laboratories. Some have objected to the test on the grounds that it utilizes fourteen tubes and therefore considerable additional work is necessary. But this, in itself, is not objectionable in view of the fact that information is afforded which is not obtainable with any other method, so far as my experience is concerned.

A rather valid objection, however, is found in the fact that sera that have stood over twenty-four hours may lose their native complement, under which circumstances the Hecht-Gradwohl technic, as outlined below, must be changed; namely, one must add sufficient complement, determined by titration, to offset the disappearance of the native complement. In a few cases where native antisheep hemolysin is absent, this, too, may be added so that these objections are thus overcome.

So far as the results of this test are concerned, as compared with the classical Wassermann, we may state that we have frequently seen a strong positive Hecht-Gradwohl reaction when a negative Wassermann was found. In our own cases which have been all checked over very carefully, undeniable history and symptoms of syphilis were obtained. It is also a fact that this test gives a positive reaction in a given case of syphilis earlier than the standard Wassermann test. It is also true that under treatment the Wassermann test becomes negative much more quickly than the Hecht-Gradwohl test. In other words, as a guide or measure of the efficacy of treatment, this test may be used with greater advantage than the classical Wassermann or many of the modern modifications.

Preparation of Glassware

The glassware is prepared in the same manner as for the Wassermann test. Great care must be exercised in cleaning the small Hecht-Gradwohl test tubes, as small quantities of blood and serum tend to remain in the tubes. This causes the tubes to become brown, or cloudy, and unfit for use.

Patient's Serum

The blood is procured in the same manner as for the Wassermann test.

After removing the serum, it is ready for use. The serum for the Hecht-Gradwohl test is not inactivated, as the complement naturally present is used instead of complement procured from guinea pig blood. The natural hemolysin present in human serum is also employed in the reaction.

Physiologic Saline

The saline solution used in the Hecht-Gradwohl test is the same as is used in the Wassermann reaction.

Antigen

Noguchi's ether-soluble, acetone-insoluble antigen is used.

After the dilution for the Wassermann reaction is determined (see titration of Noguchi's Antigen, page 2036), the antigen is diluted to double volume for use in the Hecht-Gradwohl test. For example, if the dilution for the Wassermann test is 1 part of antigen

TABLE 201

THE HECHT-GRADWOHL COMPLEMENT FIXATION TEST FOR SYPHILIS

TUBES	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Patient's serum (unheated)	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Saline	1.0 c.c.	0.9 c.c.	0.8 c.c.	0.7 c.c.	0.6 c.c.	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.2 c.c.	0.15 c.c.	0.1 c.c.	0.3 c.c.
Antigen	------	------	------	------	------	------	------	------	------	------	0.1 c.c.	0.15 c.c.	0.2 c.c.	------
Sheep cells	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.	1.0 c.c.	------	-------	------	------

Place in the water-bath at 37°–40° C. for 30 minutes.
At the end of the 30 minutes, add sheep cells to the last four tubes according to the index chart below.
Place again in the water-bath at 37°–40° C. for 30 minutes.

If the index is 0.1 c.c., 0.2 c.c., 0.3 c.c., or 0.4 c.c., Add 0.1 c.c. sheep cells to the last 4 tubes.
If the index is 0.5 c.c., 0.6 c.c., or 0.7 c.c., Add 0.15 c.c. sheep cells to the last 4 tubes.
If the index is 0.8 c.c., 0.9 c.c., or 1.0 c.c., Add 0.2 c.c. sheep cells to the last 4 tubes.
If the index is 1.1 c.c., 1.2 c.c., 1.3 c.c., or 1.4 c.c., Add 0.25 c.c. sheep cells to the last 4 tubes.
If the index is 1.5 c.c., 1.6 c.c., 1.7 c.c., Add 0.3 c.c. sheep cells to the last 4 tubes.
If the index is 1.8 c.c., 1.9 c.c., or 2.0 c.c., Add 0.35 c.c. sheep cells to the last 4 tubes.

After the first half hour in the water-bath, no further treatment of the first ten tubes is necessary. They are the index tubes; that is, the hemolytic activity of the patient's serum is determined in this titration.

Tube 14 is the control over the first ten tubes, especially when there is a low index. If Tube 14 is cloudy instead of clear, the result of the test cannot be reported. This tube does not receive antigen, and is, therefore, an anticomplementary control. If none of the first ten tubes show hemolysis after the first half hour's incubation, the serum has no natural hemolytic activity, and a Hecht-Gradwohl test cannot be made. About 2% of all bloods show no index.

to 9 parts of saline, the dilution of the same antigen for the Hecht-Gradwohl test is 1 part of antigen to 19 parts of saline. The antigen is added slowly to the saline solution. If both Wassermann and Hecht-Gradwohl tests are made on the same bloods, the antigens for the two reactions may be diluted as follows:

Make the dilution of the antigen for the Wassermann test as usual.

Add 4 c.c. of the diluted antigen to 4 c.c. saline solution. This diluted antigen solution is used in the Hecht-Gradwohl test.

Sheep Cells

The sheep cells are procured and washed in the manner described under the Wassermann reaction. For use in the Hecht-Gradwohl test, twice the strength of the suspension used in the Wassermann reaction is used.

As previously explained, since no accurate dilution of sheep cells can be made, it is better to rely on a definite color after dilution to standardize the suspension used. However, about a 6 per cent suspension of sheep cells in saline is the correct dilution.

If both Wassermann and Hecht-Gradwohl tests are made on the same bloods, the Hecht-Gradwohl sheep cell suspension can be made, then the Wassermann suspension made by adding 50 c.c. of the Hecht-Gradwohl sheep cells to 50 c.c. saline.

Water-Bath

The water-bath for the Hecht-Gradwohl test is regulated between 37° and 40° C. No inactivator is necessary for carrying out this test.

Technic of the Hecht-Gradwohl Test

All bloods for the Hecht-Gradwohl test should be examined at the same time. After recording the numbers of the bloods on the laboratory sheet, each blood is given a letter which represents its place in the day's work.

Prepare the patient's blood serum as described.

Dilute the sheep cells and the antigen.

Rack up 14 Hecht-Gradwohl test tubes for each blood serum to be tested. A tag with the letter representing the letter of the serum in the day's work is attached to the tube.

Pipette, first the serum, then the saline, then antigen in 11, 12, 13 tubes, then the sheep cells to the first 10 tubes.

If more than one test is made, place the serums in their respective test tubes first. Then add the saline to each blood serum, then the antigen, then the sheep cells.

In placing the saline in the tubes, draw up 1 c.c., and place in Tube 1. Draw up 1 c.c., and place 0.1 c.c. in Tube 10, and the remaining 0.9 c.c. in Tube 2. Draw up another 1 c.c., placing 0.2 c.c. in Tube 9, the remainder being placed in Tube 3. Continue in this manner until all tubes have received the saline.

Add the sheep cells in the same manner in order to hasten the process of pipetting. See Table 201 for quantities of reagents used.

Reading the Index

The last tube in which complete hemolysis of the sheep cells occurs is the index tube. For example, if Tubes 1, 2, 3, 4, 5, and 6 are completely hemolyzed, and the remainder show partial or complete inhibition of hemolysis, Tube 6 is the last tube in which hemolysis occurred, and is, therefore, the index tube. In such case, 0.15 c.c. sheep cells would be added to the last four tubes.

If no tubes show hemolysis, there is no index, and the test cannot be completed. Frequently, another specimen of the same patient's blood, taken at a later date, will give an index.

If all tubes show complete hemolysis, an additional 1 c.c. sheep cells is added to each of the first 10 tubes, and the rack replaced in the water-bath for another thirty minutes. If, at the end of this time, no tubes show complete hemolysis, the index is the 10th tube. or 1.0. In such case, 0.2 c.c. sheep cells should be added to the last four tubes.

If, after the addition of the extra 1.0 c.c. sheep cells to all of the first 10 tubes, and reincubation is completed, Tubes 1 and 2 show complete hemolysis, the index is 1.2, and 0.25 c.c. sheep cells is used in the test.

Reading the Results

Tube 14 should always show complete hemolysis. If it shows partial or complete inhibition of hemolysis, the result of the test is unreliable. If the blood gave a low index, then report the specimen as a ''no index'' blood. If, however, the index was above 4, some mistake has been made, and the last four tubes should be re-run.

If the specimen is negative, complete hemolysis will occur in all of the last four tubes.

A positive result may be any of the following:

Tubes	11	12	13
	+	+	+
	–	+	+
	–	–	+

If Tubes 11 and 12 are clear, and Tube 13 is cloudy, it is best to run Tube 13 over, since some error in pipetting may be responsible for this result. In treated cases of syphilis, it is not unusual to obtain such results.

Report the result of the Hecht-Gradwohl Tests as in Table 202.

TABLE 202.—RESULTS OF TESTS

TUBE 11	TUBE 12	TUBE 13	TUBE 14	REPORTS
+	+	+	–	++++
–	+	+	–	+++
–	±	+	–	++
–	–	+	–	+
–	–	–	–	–

Great care must be taken to mix the contents of the tubes thoroughly before placing in the water-bath. The tubes are narrow, and consequently, unless carefully watched, the various reagents are not thoroughly mixed with each other. If there is not enough serum to make a complete Hecht-Gradwohl test, some of the index tubes may be omitted. At least one tube in each index group should be run; for example, Tube 1, Tube 5 or 6, Tube 9 or 10. Tube 1 must always be run, as occasionally a blood will hemolyze only 0.1 c.c. sheep cells.

It is the practice of some laboratories to report as ''no index'' all bloods which show an index below 0.3 c.c.

THE CARDIOLIPIN TEST OR VDRL SLIDE TEST[1]

Reagents.—

Antigen.—

Antigen for this test is an alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and sufficient purified lecithin to produce standard reactivity (0.21% ± 0.01%). It may be purchased from Sylvana Chemical Company* or the Lederle Laboratories, Inc.† Store it in a refrigerator. Discard any antigen containing a precipitate. (See page 2016.)

Serum.—

Prepare serum in the usual manner. Heat at 56° C. for 30 minutes. Recentrifuge all sera showing particulate debris. If used more than four hours after heating, reheat at 56° C. for 10 minutes before using.

Temperature at the time of use should be 73° to 85° F.

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*407 South Jefferson St., Orange, N. J.

†30 Rockefeller Plaza, New York 20, N. Y.

[1] Harris, A., Rosenberg, A. A., Ridel, A. L.: J. Ven. Dis. Inform. 27: 169, 1946.

V.D.R.L. Slide Test Venereal Disease Research Laboratory, U.S. Marine Hospital, Staten Island 4, New York, May 1, 1946.

Buffered Saline.—

Formaldehyde, neutral, reagent grade	0.5	c.c.
Dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$)	0.093	gm.
Monobasic potassium phosphate (KH_2PO_4)	0.170	gm.
Sodium chloride A.C.S.	10.0	gm.
Distilled water	1000.0	c.c.

Store in screw-capped or glass-stoppered bottles. Reaction should be pH 6.0 ± 0.1. Temperature should be 73° to 85° F. (23° to 29° C.).

Slides.—

Slides must be fat-free. Slides to be used are glass slides with paraffin rings, or glass slides with ceramic rings. Certified Blood Donor slides or the Clay-Adams ceramic ring slides are satisfactory, provided the rings are high enough to hold the tests when the slides are rotated at the proper speed.

Antigen Emulsion.—

Pipette 0.4 c.c. buffered saline to the bottom of a 30 c.c. round, glass-stoppered or screw-capped bottle, with a *flat* bottom (on the inside of the bottle). If it is concave or convex, the first quantity of buffered saline will not cover the bottom of the bottle.* Bottles must be rinsed 8 times in tap water and twice in distilled water and then dried in an oven.

Add 0.5 c.c. antigen, drop by drop, from the lower portion of a 1.0 c.c. pipette graduated to the tip, directly onto the saline, while continuously rotating the bottle on a flat surface. Antigen is added rapidly, so that approximately 6 seconds are allowed for each 0.5 c.c. of antigen. Blow out the last drop of antigen without touching it to the saline, and continue rotation for 10 more seconds. Pipette tip should remain in the upper third of the bottle.

Proper speed of rotation is obtained when the outer edge of the bottle describes a 2-inch circle three times in a second.

Add 4.1 c.c. buffered saline, using a 5 c.c. pipette. Replace stopper and shake bottle vigorously for approximately 10 seconds, throwing the liquid from the top to the bottom of the bottle. This amount of antigen is sufficient for 250 tests. It may be used for only one day. Twice this quantity may be made at one time in a 30 c.c. bottle, but no more. Temperature for use should be 73° to 85° F. (23° to 29° C.).

Technic.—

Preliminary Testing of Antigenic Emulsion.—

Add 1 drop of antigen emulsion to 0.05 c.c. of both a reactive and a nonreactive serum, and carry out the qualitative test. Reactions should be typical. The number of antigen particles per microscopic field in the nonreactive serum should be optimum.

Antigen emulsion delivery is made with a syringe with an attached 18 gauge needle with the bevel filed off. Hold the syringe in a vertical position to deliver the proper size drop of antigen, 60 drops per c.c.

Check the needle each day. Drops should be of constant size (about $\frac{1}{60}$ c.c.). Gently shake the bottle of antigen and fill and empty the syringe once before using, to mix the antigen.

(1) VDRL SLIDE QUALITATIVE TEST

Always include reactive, weakly reactive, and nonreactive control sera.

Pipette 0.05 c.c. heated serum to one of the paraffin rings.

Add one drop (approximately $\frac{1}{60}$ c.c.) of antigen emulsion.

Rotate the slide for 4 minutes on a flat surface, describing a 2-inch circle 120 times per minute, or use the Boerner-Lukens rotator.

Read immediately, using the low power objective of the microscope, without the condenser, and the 10× ocular. Antigen particles appear as short rod forms at this magnification. Aggregation of the particles into large or small clumps is interpreted as degrees of reactivity.

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*Suitable bottles are made by Corning Glass Works, Corning, N. Y. These are No. CA-1, bottle No. 90530, Micro-reagent bottle, 30 ml., with stopper No. 14.

Report.—

Results	Report
No clumping or very slight roughness	Nonreactive (N)
Small clumps	Weakly reactive (W.R.)
Medium and large clumps	Reactive (R)

The *weakly reactive* range includes clumpings similar to those recorded as plus-minus or 1 plus, the *reactive* range, those reported 2 plus, 3 plus, or 4 plus, in other slide tests. This method of reporting is not recommended.

Zonal reactions, due to excess of reactive serum component, appear as irregular clumps, loosely bound. Reactive results show large or small clumps, fairly uniform in size. If zonal reactions are suspected, dilute the serum 1:5 and 1:25, and re-test. The maximum reaction in either dilution, if greater than that with undiluted serum, is the test finding. ✓

(2) VDRL SLIDE QUANTITATIVE TEST A

All sera producing reactive or weakly reactive results in the qualitative VDRL slide test should be tested quantitatively.

Dilute the serum 1:10 by adding 0.1 c.c. of the heated serum to 0.9 c.c. of 0.9% saline, using a 0.2 c.c. pipette graduated in 0.01 c.c. for measuring the serum.

Mix the serum and saline thoroughly, and allow the pipette to stand in the test tube.

Using this same pipette, transfer 0.05, 0.02, and 0.01 c.c. quantities of the 1:10 dilution to the 4th, 5th, and 6th rings, respectively, of the VDRL test slide.

With the same pipette, transfer 0.05, 0.02, and 0.01 c.c. quantities of the undiluted serum into the 1st, 2nd, and 3rd ringed areas.

Add 1 drop (0.03 c.c.) of 0.9% saline to the 2nd and 5th rings of each serum by vertical delivery, from a 15 gauge hypodermic needle fitted to a glass syringe. Needles should be checked for proper drop size.

Add 1 drop (0.04 c.c.) of 0.9% saline to the 3rd and 6th rings by vertical delivery from the syringe fitted with a 13 gauge needle.

The 6 mixtures are equivalent to dilutions of 1:1 or undiluted, 1:2.5, 1:5, 1:10, 1:25, and 1:50.

Rotate the slides gently by hand for 15 seconds to mix the serum and saline.

Add 1 drop ($\frac{1}{60}$ c.c.) of antigen emulsion to each ring, using a syringe and needle as described for the slide qualitative serum test.

Rotate the slides in the manner described on page 2063.

Read the results microscopically. The highest serum dilution giving a reactive result (not weakly reactive) is reported as the reactivity end point; e.g., reactive–1:25 dilution, or reactive–25 dils.

If all the dilutions give reactive results prepare a 1:100 dilution of that serum by adding 0.1 c.c. of the 1:10 serum to 0.9 c.c. of 0.9% saline solution and proceed as above. The serum dilutions are now 1:100, 1:250, and 1:500. Test each dilution and report.

(3) VDRL SLIDE QUANTITATIVE TEST B

Prepare a 1:8 dilution of the serum by adding 0.1 c.c. of heated serum to 0.7 c.c. of 0.9% saline, using a 0.2 c.c. pipette graduated in 0.01 c.c. for measuring the serum.

Mix the serum and saline solution thoroughly and allow the pipette to stand in the test tube.

Using this pipette, transfer 0.04, 0.02, and 0.01 c.c. of the 1:8 serum dilution into the 4th, 5th, and 6th paraffin rings, respectively.

With the same pipette, transfer 0.04, 0.02, and 0.01 c.c. of the undiluted serum into the 1st, 2nd, and 3rd rings.

Add 2 drops (0.01 c.c. in each drop) of 0.9% saline to the 2nd and 5th rings of each serum by vertical delivery from a 23 gauge hypodermic needle fitted to a glass syringe.

Add 3 drops of 0.9% saline to the 3rd and 6th rings of the serum.

Rotate the slides gently by hand for 15 seconds to mix the serum and saline.

Add 1 drop ($\frac{1}{75}$ c.c.) of antigen emulsion to each ring, using a syringe and needle of appropriate size.

Complete tests as described above. Serum dilutions are 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32.

If all dilutions give reactive results continue with the dilutions and tests.

Reading and Reporting VDRL Slide Quantitative Tests

Read microscopically at 100× magnification.

Report results in terms of the greatest serum dilution that produces a reactive (not weakly reactive) result in accordance with Table 203.

TABLE 203

DILUTIONS OF SERUM					REPORT
				Method A	
1:1	1:2.5	1:5	1:10	1:25	
R	WR	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	WR	N	N	Reactive, 1:2.5 dilution, or 2.5 dils.
R	R	R	RR	N	Reactive, 1:5 dilution, or 5 dils.
				Method B	
1:1	1:2	1:4	1:8	1:16	
R	WR	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	WR	N	N	Reactive, 1:2 dilution, or 2 dils.
R	R	R	WR	N	Reactive, 1:4 dilution, or 4 dils.

R—reactive; WR—weakly reactive; N—nonreactive.

NOTE: Under conditions of high temperature and low humidity, antigen emulsions may be stored in a refrigerator but should be restored to room temperature before use. To prevent surface drying under these conditions, tests should be completed and read as rapidly as possible.

THE VDRL TUBE FLOCCULATION TEST

General Equipment.—

Kahn shaking machine, which must be operated at 275 to 285 oscillations per minute.

Reading lamp, fluorescent or gooseneck type.

Reagents.—

Antigen.—

VDRL slide flocculation test antigen (see page 2062).

Saline Solutions.—

Prepare **1% buffered saline** as for the VDRL slide flocculation test (page 2063).

Prepare **unbuffered 1% sodium chloride solution** by adding 1 gm. of dry sodium chloride to each 100 c.c. of distilled water.

Serum.—

Use clear serum obtained by centrifuging whole clotted blood. Heat at 56° C. for 30 minutes or at 60° to 62° C. for 3 minutes before testing. Examine all sera when they are removed from the water-bath. Recentrifuge those which contain particulate debris. Sera to be tested more than 4 hours after being heated should be reheated at 56° C. for 10 minutes just before use.

Antigen Emulsion.—

Prepare antigen emulsion as described for the VDRL slide flocculation test (page 2063).

Add 4 parts of 1% sodium chloride solution to 1 part of VDRL slide test emulsion.

Mix well and allow to stand 5 minutes or more, but no longer than 2 hours, before use. This solution will be referred to as "**diluted antigen emulsion.**"

(1) QUALITATIVE SERUM TEST

Pipette 0.5 c.c. of heated serum into a test tube 12 by 75 mm. outside diameter.

Add 0.5 c.c. diluted antigen emulsion to each serum.

Shake tubes in a Kahn shaker for 5 minutes.

Centrifuge all tubes for 10 minutes at 2,000 r.p.m. in No. 1, or at 1,700 r.p.m. in No. 2, I.E.C. centrifuge (or its equivalent).

Return tubes to the Kahn shaking machine and shake for exactly 1 minute.

Include reactive and nonreactive sera controls.

Reading and Reporting Qualitative Test.—

Read reactions *as soon as secondary shaking period is completed,* by holding the tubes close to the shade of a reading lamp with a black background, at approximately eye level. A shaded fluorescent desk lamp or a gooseneck-type lamp with a blue bulb is a satisfactory reading light source.

Record results as follows:

Reactive—

Visible aggregates in a clear or slightly turbid medium. All borderline reactions, where the observer is in doubt regarding visible clumping, should be reported as negative.

Nonreactive—

No visible clumping or aggregation of antigen particles; appearance slightly turbid or granular; definite silken swirl on gentle shaking.

Note: Cloudy or hemolyzed sera may cause completed tests to be too turbid for macroscopic reading. Fluid from these tubes may be examined microscopically. Reactive reports may be rendered when large antigen-particle masses are detected microscopically if the serum tested is found to be free of particles at the same magnification.

Zonal reactions, due to excess of reactive serum component, may appear to be very weak, or, in rare instances, nonreactive. Whenever a zonal reaction is suspected, another test should be performed using 0.1 c.c. of heated serum and 0.4 c.c. of saline in place of the original 0.5 c.c. of serum. If a reactive result is obtained with the smaller amount of serum, a reactive report should be issued.

(2) QUANTITATIVE SERUM TEST

Pipette 0.5 c.c. of freshly prepared 0.9% saline into each of 5 or more test tubes (12 by 75 mm.).

Add 0.5 c.c. of heated serum to the first tube and mix.

Transfer 0.5 c.c. from the first to the second tube and mix.

Continue transferring 0.5 c.c. from each tube to the next, and mixing, until the last tube is reached.

Discard 0.5 c.c. from the last tube.

Add 0.5 c.c. of diluted antigen emulsion to each tube and proceed as described under "VDRL Slide Qualitative Serum Test," page 2063.

Reading and Reporting Quantitative Test.—

The greatest dilution of serum which produces a definitely positive reaction is the reactivity end point in accordance with the following example:

SERUM DILUTIONS					REPORT
(1:1)	1:2	1:4	1:8	1:16	
R	N	N	N	N	Reactive,undiluted only, or 1 dil.
R	R	R	N	N	Reactive, 1:4 dilution, or 4 dils.
R	R	R	R	N	Reactive, 1:8 dilution, or 8 dils.

THE VDRL TEST ON SPINAL FLUID

Reagents.—

The VDRL slide test antigen and the 1% buffered saline used in this test are the same as for the VDRL slide flocculation test (pages 2062, 2063).

10% Sodium Chloride Solution.—

Dissolve 10 gm. dry sodium chloride (A.C.S.).
in 100 c.c. distilled water.

Filter through filter paper.

Spinal Fluid.—

Centrifuge each spinal fluid and decant the supernatant fluid. Spinal fluids which are visibly contaminated or which contain gross blood are unsatisfactory for this test.

Heat in a water bath at 56° C. for 15 minutes, and cool to room temperature before testing.

Sensitized Antigen Emulsion.—

Prepare antigen emulsion as for the VDRL slide flocculation test, page 2063.

Add 1 part of 10% sodium chloride solution
to 1 part of VDRL slide test emulsion.

Mix well and allow to stand at least 5 minutes but not more than 2 hours before using.

QUALITATIVE SPINAL FLUID TEST

Pipette 1 c.c. heated spinal fluid into a 13 by 100 mm. test tube.

At the same time, prepare similar tubes, but using reactive and nonreactive spinal fluid specimens as controls.

Resuspend the sensitized antigen emulsion immediately before use by inverting the container several times.

Add to each tube in the test and to each control 0.2 c.c. of sensitized antigen emulsion.

Shake the racks of tubes on a Kahn shaking machine for 15 minutes.

Centrifuge for 5 minutes at 1,800 r.p.m. if a No. 1 centrifuge is used, and at 1,600 r.p.m. if a No. 2 is used.

Shake tubes in a Kahn shaking machine for *exactly* 2 minutes.

Read reactions as soon as possible after the second shaking period. Hold tubes close to the shade of a desk lamp with a black background. Each tube may be held motionless or shaken gently during the reading. Excessive agitation should be avoided.

Report as follows:

Reactive: definitely visible aggregates suspended in a water-clear or turbid medium.

Nonreactive: no aggregation, complete dispersion of particles, appearance turbid or slightly granular.

QUANTITATIVE SPINAL FLUID TEST

Prepare dilutions of spinal fluid, 1:2, 1:4, 1:8, 1:16, 1:32, etc., as follows.

Pipette 1.0 c.c. of 0.9% sodium chloride solution into each of 5 or more tubes.

Add 1.0 c.c. of heated spinal fluid to tube 1.

Mix and transfer 1.0 c.c. to tube 2.

Mix and transfer 1.0 c.c. to tube 3.

Continue mixing and transferring 1.0 c.c. to each successive tube. Discard 1.0 c.c. from the last tube.

Test each dilution as for the qualitative spinal fluid test.

Read each tube as for the qualitative spinal fluid test.

Report as follows:

Report test results in terms of the highest dilution of spinal fluid producing a reactive result. The term "dils," which expresses the same dilution reactivity end point, may be applied. Example:

SPINAL FLUID DILUTIONS					REPORT
1:1	1:4	1:8	1:16	1:32	
N	N	N	N	N	Reactive, undiluted only, or 1 dil.*
R	R	R	N	N	Reactive, 1:8 dilution, or 8 dils.
R	R	R	R	N	Reactive, 1:16 dilution, or 16 dils.

*Reactive finding with undiluted spinal fluid in the qualitative test.

THE KAHN PRECIPITATION TEST FOR SYPHILIS

Materials Required for Kahn Test for Syphilis

The material for thirty tests is given.

About 12 antigen vials. These are flat bottom vials, 5.5 cm. in length and 1.5 cm. inside diameter. One gross Kahn test tubes, 7.5 cm. in length and 1 cm. inside dimeter. (These are made of pure white glass.)

6 10 c.c. pipettes, graduated in 0.1 c.c.

3 dozen serologic pipettes, 1 c.c., graduated in 0.01 c.c. to the tip.

2 0.5 c.c. pipettes graduated to 0.025 c.c. and

2 0.25 c.c. pipettes graduated to 0.0125 c.c. These are Kahn antigen suspension pipettes and should be graduated to tip.

2 0.2 c.c. pipettes graduated in 0.001 c.c.

3 Kahn test tube racks. These racks have three 10 hole rows. The holes are 0.5 inch in diameter. The center row of holes is offset 0.5 inch. The rack is 3 inches wide, 11½ inches long and 2¾ inches high.

1 shaking machine, 3 or 6 rack capacity. Speed of 275 to 285 oscillations per minute with a stroke of 1½ in.

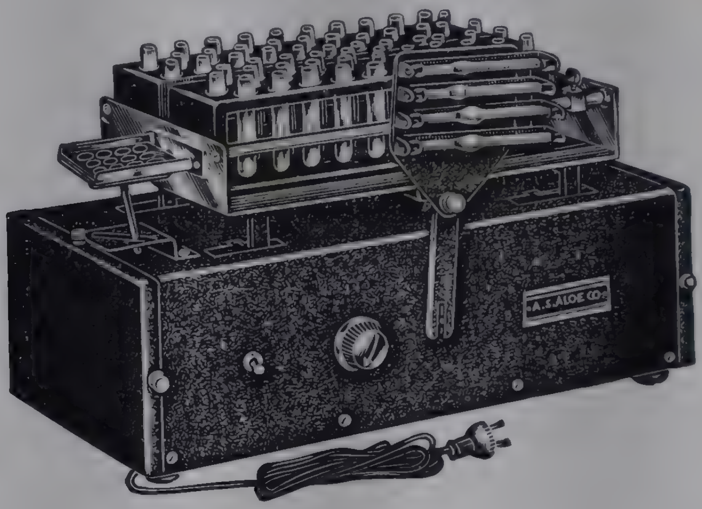

Fig. 604.—Aloe-Feder shaking machine. (Courtesy A. S. Aloe Co., St. Louis, Mo.)

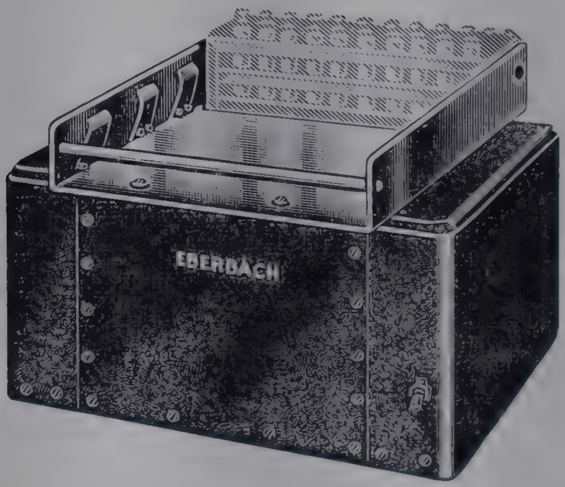

Fig. 605.—Kahn shaking machine. (Courtesy Eberbach Corporation, Ann Arbor, Mich.)

3 1,000 c.c. Pyrex Erlenmeyer flasks for saline solutions.

1 56° C. automatic temperature control water bath. If complement fixation tests are made in the same laboratory, the same bath may be used.

1 centrifuge.

Numerous test tubes for collecting the blood and removing the serum.

1 gooseneck lamp.

1 microscope mirror.

Preparation of Glassware

Rinse the glassware thoroughly in tap water.

Place new glassware in 1% hydrochloric acid solution for two hours, otherwise omit this step.

Rinse thoroughly in tap water.

Rinse thoroughly in distilled water.

Sterilize in the dry heat sterilizer at 170° C. for two hours. (It is not necessary actually to sterilize; glassware needs only to be thoroughly dry.)

This should be done about once a month: The pipettes should be kept overnight in cleaning solution (page 192), then rinsed thoroughly in tap water. Place them in a crock, and wash in running tap water for several hours.

Rinse in distilled water.

Sterilize in the dry heat oven at 170° C. for two hours (not necessary to sterilize, only to dry thoroughly).

The glassware must be neutral and dry.

Saline

The saline is prepared in the same manner as for the Wassermann test except that 0.9% sodium chloride is used. If any traces of foreign matter remain in the solution, they should be removed by filtration even after sterilization. The sterility of the solution is not as important as its chemical purity and its freedom from particles. Saline should be prepared every two weeks.

Patient's Serum

The patient's serum is obtained and prepared in the same manner as for the Wassermann reaction. If sera are held over from day to day, they should be reheated for ten minutes at 56° C. before use. Tests should be made within 5 to 10 minutes after removing serum from the bath. If a sediment develops, it should be removed by centrifuging. Any trace of cells must also be removed. The serum must be clear. Hemolyzed blood may be used for the Kahn test, provided it contains no precipitate.

Antigen

Antigen already titrated and standardized, plain or cardiolipin type, with the titer for serum and spinal fluid, may be purchased from various biological houses. It should be stored at room temperature in the dark.

To make the antigen, purchase powdered beef heart (Digestive Ferments Co.). Place 25 grams of powdered beef heart in a 250 c.c. Pyrex Erlenmeyer flask.

Add 100 c.c. anesthesia ether.

Shake the flask at frequent intervals for ten minutes.

Filter through filter paper in a Buckner funnel fitted to a side-arm filter flask by means of a one-hole rubber stopper.

Transfer the moist beef heart to the original flask.

Add 75 c.c. ether, and again shake for ten minutes at frequent intervals.

Refilter, and again discard the filtrate. Use fresh filter paper.

Transfer the moist heart to the flask, and again add 75 c.c. ether.

Shake for ten minutes, and filter through filter paper.

Repeat this process for the fourth extraction with ether. Use fresh filter paper for each filtration.

Spread the moist heart muscle on a sheet of white paper in the incubator. Allow it to dry. It should be free from the odor of ether before the following procedures are carried out.

Weigh the dry powder. Place in a 250 c.c. Erlenmeyer flask.

Add 5 c.c. 95% alcohol for every gram of powder.

Cover a cork with tin foil and place tightly in the flask.

Shake the flask for 10 minutes.

Allow to remain at room temperature in the dark for 3 days. Do not shake the flask during this period.

Filter through filter paper, shaking the flask for five minutes before filtration. Place the filtrate in a ground glass stoppered bottle in the dark at room temperature.

This is the **stock antigen solution.**

For cholesterolization add to every 1 c.c. stock antigen solution 6 mg. cholesterol.

Place the bottle or flask in a warm bath until all the cholesterol is in solution. Do not keep this solution in the refrigerator, as the cholesterol will not remain in solution.

After the cholesterol is dissolved, filter through filter paper until absolutely clear. The cholesterolized antigen must be titrated before use.

STANDARDIZATION OF KAHN ANTIGEN*

(a) Determination of Titer

The purpose of the titration is to determine the minimum amount of 0.9% saline to be added to 1 c.c. of antigen to produce a suspension of aggregates that completely disperses upon the addition of a designated amount of saline solution, and which reproduces the opalescence of the standard antigen.

Using a 1 c.c. pipette graduated in 0.01 c.c., place 0.9, 1.1, 1.2, 1.3, 1.4, 1.5, 1.7, and 1.9 c.c., respectively, of 0.9% saline in 8 antigen-suspension vials.

Using a 1 c.c. pipette, place 1 c.c. of 0.6% cholesterolized antigen to be titrated in each of 8 similar vials. Set up a standard antigen control at the same time at the titer indicated on the label.

Prepare the different antigen suspensions by mixing the antigen with the varying amounts of saline. Empty the saline into the antigen as rapidly as possible without waiting to drain the tube, and pour the mixture back and forth 12 times. Allow to stand for 30 minutes.

Test for dispersibility in saline of the lipid aggregates in the antigen-saline solution suspension as follows:

1. Set up 9 series of 3 Kahn tubes each.
2. Pipette 0.05, 0.025, and 0.0125 c.c. quantities of each of the 8 antigen suspensions, after thorough agitation, to the bottoms of the tubes, in series, using a 0.25 c.c. antigen pipette. Pipette the same quantities of emulsion, prepared from the standard antigen, into the last 3 tubes.

   Use a different antigen pipette for each suspension.
3. Using a 1 c.c. pipette, add 0.15 c.c. saline solution to each of the 27 tubes.
4. Shake the racks vigorously for 10 seconds by hand and then for 3 minutes on a Kahn shaking machine at a speed of 275 to 285 oscillations per minute.
5. Add 1 c.c. of saline to the tubes containing the 0.05 c.c. amounts of antigen suspension, and 0.5 c.c. to the remaining tubes. Shake the rack by hand to mix and observe whether the mixtures are opalescent or contain aggregates.

A typical titration may show cloudiness in the 3-tube test containing the antigen suspension which was prepared with 0.9 c.c. and with 1.1 c.c. saline. These suspensions contain aggregates which were not completely redispersed in saline solution. Those containing antigen suspension mixed with 1.3 c.c. of saline may appear opalescent, like the antigen control. The other four tests may appear clearer than the control. In this case, the titer of the antigen would be 1 c.c. of antigen plus 1.3 c.c. of saline; that is, 1.3 c.c. is the smallest amount of saline solution, which, when added to 1 c.c. of antigen, produces aggregates capable of complete dispersion upon the further addition of saline solution and which reproduces the opalescence of the standard antigen.

Table 204 illustrates a typical antigen titration. The ''normal'' range of titers for standard antigen may vary from 1 + 1.1 to 1 + 1.5.

TABLE 204.—TYPICAL ANTIGEN TITRATION

ANTIGEN PLUS 0.9% SALINE SOLUTION	APPEARANCE OF MIXTURE
1 + 0.9	Cloudy, nondispersible aggregates
1 + 1.1	Slightly cloudy, fine, nondispersible aggregation
1 + 1.2	Very slightly cloudy
1 + 1.3	Opalescent, standard (titer)
1 + 1.4	Very slightly clearer
1 + 1.5	Slightly clearer
1 + 1.7	Very clear
1 + 1.9	Almost water clear
Standard antigen control: Opalescent, standard	

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*Serologic Tests for Syphilis, 1955 Manual, U.S. Department of Health, Education, and Welfare, Public Health Services, Publication No. 411.

Antigens made with progressively increasing volumes of saline solution will usually show a correspondingly progressive increase in clarity of mixtures. Rarely, however, an antigen suspension will show a ''titration zone''; that is, cloudiness and nondispersible aggregates may appear again beyond the titer, for example, in tubes containing 1.7 or 1.9 c.c. of saline. This antigen has a short workable range and should not be used.

(b) Determination of Reactivity Level

A series of sera with graded reactivities may be prepared from pooled, Seitz-filtered reactive and nonreactive sera by combining selected proportions of these sera so that the desired number of strongly and weakly reacting specimens is obtained. These may be used for preliminary testing but not for final checking of the reactivity. All sera should be heated before use.

(1) **Preliminary Testing.—**

Prepare antigen suspensions with both antigens according to their respective titers. Allow to stand for 10 minutes.

Carry out tests with at least 10 sera as for the Kahn standard qualitative test with serum. If comparable reactions are obtained with both antigen suspensions, final checking should be made.

(2) **Final Checking of Antigen.—**

Obtain at least 50 sera that show varying degrees of reactivity in the Kahn test, and 50 nonreactive sera.

Perform Kahn standard qualitative tests, using both antigens simultaneously on all the sera.

Each serum should be tested with both antigens in the same rack so as to eliminate the time factor. Two readings should be made in each instance where recordable reactions are observed during the first reading. The two readings are necessary since some antigens that produce standard first readings may allow readable particles to redisperse at a rate different from that of the standard antigen. Thus, the final report on a given specimen could be less or greater than that obtained with standard antigen. If the results produced by the new antigen are comparable with those of the standard antigen, it is considered to have standard reactivity.

(3) **Correction of Antigen.—**

The reactivity level may be greater or less than that of the standard antigen. Correction to standard requirements can usually be accomplished.

TABLE 205.—STANDARDIZATION OF KAHN STANDARD ANTIGEN

ANTIGEN REACTIVITY	METHOD OF ADJUSTMENT
Less reactive than standard	1. Addition of cholesterolized alcohol 2. Addition of sensitizing reagent 3. Addition of sensitizing reagent plus cholesterolized alcohol 4. Addition of overreactive antigen
More reactive than standard	1. Addition of cholesterolized alcohol 2. Addition of a less reactive antigen 3. Lecithin correction solution

Cholesterolized Alcohol:

To 100 c.c. of 95% alcohol in a 250 c.c. glass-stoppered Erlenmeyer flask or bottle, add 600 mg. of cholesterol. Rotate the flask in a warm water bath until all cholesterol is dissolved. Filter when cool.

Sensitizing Reagent:

Refilter the ether filtrate obtained in the preparation of the antigen to remove traces of powdered muscle.

Place in an evaporating dish and evaporate with the aid of an electric fan. A few c.c. of water may condense in the evaporating dish, and appear at the bottom of the dish. Remove with a capillary pipette as it is formed. The lipid residue is brownish, semitransparent, and viscous.

When the volume has been reduced to a point where ether odor is no longer detectable, transfer the residue to a small, previously weighed evaporating dish, and weigh.

Add a volume of absolute alcohol equal to 10 c.c. per gram of residue. Transfer the lipid mixture to a flask.

Extract for 30 minutes at room temperature, with frequent shaking of the flask. Little of the residue is soluble in alcohol, lipid masses being distributed throughout the mixture.

Place in a refrigerator at 4° to 9° C. for 3 hours.

Filter while cold.

Place the flask containing the clear filtrate in an incubator at 37° C. for 24 hours.

Allow the clear filtrate to stand for 3 days at room temperature. If a precipitate forms, the solution should be refiltered.

Cholesterolize the filtrate with 6 mg. of cholesterol per c.c., according to the usual technic.

The cholesterolized extract is known as the sensitizing reagent. Filter. It is now ready for use. Keep in the dark at room temperature.

Correction of Antigens More Reactive Than Standard Antigens:

(1) *Mix the more reactive antigen with a less reactive antigen.*

a. Add equal amounts of the more reactive and less reactive antigens to a small bottle and mix.

b. Titrate, as described on page 2070.

c. Make preliminary comparative tests at the determined titer. Use a standard antigen as control.

d. If results are comparable, perform a final check, page 2071.

e. If antigen reactivity is not comparable, try different proportions of the two antigens.

f. Perform a final check after the entire lot of antigen has been corrected.

(2) *Add lecithin correction solution to the antigen.*

Prepare a **stock lecithin correction solution** from egg lecithin:

a. Add 5 gm. commercial egg lecithin to 100 c.c. of 0.6% cholesterolized absolute alcohol.

b. Extract 1 hour at 56° C. in a water bath.

c. Cool and filter off clear solution.

Method:

a. Add a given amount, such as 0.2 c.c., of the stock lecithin solution to 10 c.c. of the overreactive antigen.

b. Titrate the antigen, page 2070.

c. Test this antigen with 10 sera, most of which are known to give weakly reactive results, using standard antigen as a control.

d. If the modified antigen gives results comparable to those obtained with the standard antigen, adjust the entire amount of the more reactive antigen, and submit to final checking.

(3) *Dilute with cholesterolized alcohol.*

Refer to ''Corrections of antigens less reactive than the standard,'' below.

Corrections of Antigens Less Reactive Than Standard Antigen:

(1) *Dilution of antigen with cholesterolized alcohol.*

a. Add 10 c.c. of the less reactive antigen (0.6% cholesterolized) to a ½-ounce bottle. Add 1 c.c. of the 0.6% cholesterolized alcohol.

To another bottle of the same size, add 10 c.c. of the 0.6% cholesterolized antigen and 2 c.c. of 0.6% cholesterolized alcohol, making 10 and 20% dilutions, respectively.

b. Titrate both according to the method on page 2070.

c. Make comparative tests with weakly reactive sera, using standard antigen simultaneously as a control.

d. If neither 10 nor 20% dilution brings the antigen to standard reactivity, try other dilutions not exceeding 30%.

(2) *Addition of sensitizing reagent.*

Some less reactive antigens can be brought to standard level of reactivity by adding a trace of sensitizing reagent (page 2071), 0.2% to 0.7%. In some cases, sensitizing reagent in addition to dilution with cholesterolized alcohol is necessary.

(3) *Mixing less reactive antigen with one of greater reactivity.*

This is the same as described above for correction of a more reactive antigen by mixing with a less reactive one.

CHARACTERISTICS OF DIFFERENT KAHN STANDARD ANTIGENS AND THEIR IMPORTANCE IN SEROLOGIC RESULTS

Kahn antigens may show ''sloping'' or ''flat'' titers.

Antigens with sloping titrations show titration pictures of increasing clarity with the increase in the amounts of saline solution used in the preparation of the suspensions.

Antigens with flat titrations show titration pictures of similar clarity with the increase in the amounts of saline solution used in the preparation of the suspensions.

Antigens with a sloping titer are the most desirable. Some antigens which show a flat titer before standardization may show a sloping titer after standardization. Antigens which continue to show a flat titer after standardization are safe for use provided they do not begin to show a ''reversed'' sloping titration, namely, increased cloudiness following an increase in the amount of saline solution added to the antigen.

REFERENCES

Kahn, R. L.: Technique of Standard Kahn Test and of Special Kahn Procedures, revised and enlarged edition, 1945, University of Michigan Press.

Kahn, R. L.: Serology With Lipid Antigens, Baltimore, 1950, Williams & Wilkins Co.

Serologic Tests for Syphilis, 1955 Manual, Public Health Service Publication No. 411 U.S. Department of Health, Education, and Welfare, Public Health Service.

The Standard Kahn Qualitative Precipitation Test of Blood Serum

Kahn racks should be used.

All sera for the Kahn test should be prepared at the same time. After recording, each serum is given a letter which represents its place in the day's work.

Centrifuge the bloods for 10 minutes at 2,000 r.p.m. and remove the serum.

Heat serum for 30 minutes at 56° C. Allow to stand at room temperature at least 10 minutes.

Prepare a *dilution of the antigen* as follows:

Place 1 c.c. antigen in an antigen vial.

Place 1 c.c. (or the quantity indicated by the titer) of saline in another antigen vial.

Mix the two by pouring back and forth twelve times without waiting to drain the tubes, pouring the saline into the antigen first.

Wait at least 10 minutes, and not longer than 30 minutes, before beginning the tests. Protect with waxed paper and shake briskly, to suspend the particles, placing the thumb over the waxed paper.

Use a 1 c.c. pipette graduated in 0.01 c.c. for measuring the 0.05 c.c. quantities of antigen suspension into the first row.

Use a 0.2 c.c. pipette graduated in 0.001 c.c. or Kahn antigen pipette for measuring the other quantities of antigen suspension.

Use the back row of tubes for the 0.0125 c.c. quantities of antigen suspension.

Draw up a pipetteful of antigen suspension. Remove any excess antigen from the outside with a clean cloth. Hold the pipette in a vertical position. Place it into each tube to the bottom of the tube. Place 0.05 c.c. of antigen suspension in the front row, 0.025 c.c. in the middle row, and 0.0125 c.c. in the back row of tubes.

Use a 1 c.c. pipette graduated in 0.01 c.c. for measuring the serum. Use a different pipette for each serum.

Place 0.15 c.c. serum in each of its three tubes.

Be sure to make a test on a known reactive and a known nonreactive serum (controls), and if possible on sera giving varying degrees of reactivity.

After placing the serum in each tube, shake the rack vigorously by hand for 10 seconds. Let stand 3 to 7 minutes at room temperature, and then place in the shaking machine and shake for 3 minutes.

Place 1 c.c. saline in each of the front tubes.

Add 0.5 c.c. saline to each of the tubes in the middle and back rows.

Mix by shaking vigorously. Read the results at once and check them 15 minutes later. Complete the addition of antigen suspension and sera to one rack before adding antigen suspension and sera to another rack. Add saline to one rack and complete reading before adding saline to another rack.

Reading Results

Place a microscope mirror on the laboratory table with the concave side upward.

Adjust a reading lamp above the mirror so that the bulb image is not visible. The Kahn tube is to be held within the cone of light. Adjust light properly.

Place each tube to be read in a nearly horizontal position, with the lower portion of the tube about 1 or 2 inches above the mirror, and note the degree of flocculation by viewing the image of the tube contents in the mirror.

Recording Results.—

Record degree of flocculation as follows:

4+	Relatively large floccules
3+	Medium-sized floccules
2+	Fine floccules easily distinguishable
1+	Very fine floccules
±	Extremely fine floccules, just distinguishable
–	An opalescent medium free from visible floccules

Reporting Results.—

There are two types of reactions possible with the Kahn test, the typical reactions and the zonal reactions. The typical reactions are characterized by greatest degree of flocculation with the smallest amounts of antigen, while the zonal reactions are characterized by having the greatest degree of flocculation with the largest amounts of antigen or equal degree with all three amounts of antigen suspension.

(a) *Typical Reactions.*

Add the pluses, disregarding the ± readings, obtained in the first and second readings. Report results as reactive, weakly reactive, and nonreactive according to Table 206.

TABLE 206.—REPORTING TYPICAL KAHN REACTIONS

SUM OF PLUSES OF FIRST AND SECOND TUBE READINGS	REPORT
22 to 24	Reactive (4+)
16 to 21	Reactive (3+)
10 to 15	Reactive (2+)
5 to 9	Weakly reactive (1+)
4*	Weakly reactive (±)
3 or less†	Nonreactive

*Should be given a nonreactive report when results are – 1 1 and – 1 1 on first and second readings.

†Should be given a weakly reactive report when the results are – – 3 on first reading and – – ± on second reading, or when they are – – 2 on first reading and – – 1 on second reading.

(b) *Zonal Reactions.*

Report zonal reactions according to Tables 207 to 213.

TABLE 207.—ZONAL REACTIONS, REACTIVE 4+, SUPPLEMENTARY TESTS NOT NECESSARY

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	4	4	3	4	4	3
2	4	4	2	4	4	2
3	4	4	1	4	4	1
4	4	4	–	4	4	–

Table 208.—Zonal Reactions, Reactive 4+, If Supplementary Tests 1 and 2 Are Reactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	4	3	3	3	3	3
2	4	3	2	4	3	2
3	4	3	–	4	3	–
4	4	1	–	4	–	–
5	3	3	3	3	3	3
6	2	2	2	2	2	2
7	2	–	–	2	–	–
8	1	1	1	1	1	1
9	1	–	–	1	–	–
10	±	±	±	±	±	±
11	±	–	–	±	–	–

Table 209.—Zonal Reactions, Reactive 3+, If Supplementary Tests 1 and 2 Are Nonreactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	4	3	3	3	3	3
2	4	3	2	4	3	2
3	3	3	3	3	3	3

Table 210.—Zonal Reactions, Reactive 2+, If Supplementary Tests 1 and 2 Are Nonreactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	4	2	2	3	2	2
2	4	1	1	4	1	1
3	4	3	±	4	3	±
4	3	3	2	3	3	–

Table 211.—Zonal Reactions Weakly Reactive (1+) If Supplementary Tests 1 and 2 Are Nonreactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	4	1	±	3	1	±
2	3	2	±	3	–	–
3	3	±	–	3	±	–
4	2	2	2	2	2	2

Table 212.—Zonal Reactions Weakly Reactive (±) If Supplementary Tests 1 and 2 Are Nonreactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	2	2	1	2	2	±
2	2	2	–	2	1	–
3	2	1	–	2	±	–

Table 213.—Zonal Reactions Nonreactive If Supplementary Tests 1 and 2 Are Nonreactive

SERUM NO.	FIRST READING			SECOND READING		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	2	±	±	2	±	±
2	2	±	±	2	–	–
3	2	±	–	2	–	–
4	2	–	–	2	–	–
5	1	1	±	1	±	±
6	1	±	±	1	–	–
7	±	±	±	±	–	–
8	±	–	–	±	–	–

Supplementary Test No. 1

Place 0.05 c.c. antigen suspension in each of two tubes.

Add 0.05 c.c. of previously heated serum to tube 1.

Add 0.1 c.c. of serum to tube 2.

Shake the rack by hand for 10 seconds. Allow to stand 3 to 7 minutes at room temperature.

Shake the rack on a Kahn shaker for 3 minutes.

Add 1 c.c. of saline to each tube.

Shake by hand to mix and read immediately, and again after 15 minutes.

Reactive: 4+ or 3+ reaction in each of the two tubes.

Nonreactive: Less than 3+ reaction in each of the two tubes.

Supplementary Test No. 2

Prepare serum dilutions 1:4, 1:8, and 1:16.

Place 0.6, 0.4, and 0.4 c.c. of saline in tubes numbered 1, 2, and 3, respectively.

Add 0.2 c.c. of serum to tube 1 and mix.

Transfer 0.4 c.c. from tube 1 to tube 2 and mix.

Transfer 0.4 c.c. from tube 2 to tube 3, and mix.

Add 0.0125 c.c. of antigen suspension to the bottom of three Kahn tubes numbered 1, 2, 3, respectively.

Add 0.15 c.c. of the 1:4, 1:8, and 1:16 serum dilutions to tubes 1, 2, and 3, respectively, beginning with the highest serum dilution.

Shake the rack by hand 10 seconds to mix. Allow to stand 3 to 7 minutes.

Shake in a Kahn shaker for 3 minutes.

Add 0.5 c.c. saline to each tube and shake to mix.

Read immediately.

Reactive: 4+ or 3+ reaction in at least the 1:4 dilution.

Nonreactive: 2+ reaction or less in the 1:4 and higher dilutions.

Reporting Results

If the supplementary tests 1 and 2 are reactive, report results of the standard test as reactive, 4+.

If supplementary tests 1 and 2 are nonreactive, report results according to Tables 209 through 213.

If supplementary test 1 is reactive and supplementary test 2 is nonreactive, prepare 1:32, 1:64, and 1:128 dilutions of the serum and test as described under supplementary test 2. If the result is reactive in one or more tubes (4+ or 3+ reaction), report the results of the standard test as reactive 4+. If the result is nonreactive in all tubes, less than 3+ reaction, report the results of the standard test as indicated in Tables 209 through 213.

If supplementary test 1 is nonreactive and supplementary test 2 is reactive, test the undiluted serum in a 1:2 ratio of serum to antigen suspension (0.05 c.c. of antigen plus 0.025 c.c. serum). If the result is reactive (4+ or 3+), report the results of the standard test as reactive 4+. If the result is nonreactive (less than 3+), report the results of the standard test as indicated in Tables 209 through 213.

Control System for Kahn Standard Test

Control sera of graded reactivity and a saline solution control should be tested with each antigen suspension along with the regular tests.

Do not report results on sera or spinal fluids if the prescribed pattern of reactivity is not obtained with the controls. Failure to obtain satisfactory results may be caused by (1) improperly prepared saline solution; (2) substandard antigen; (3) incorrectly prepared antigen suspension; (4) using antigen suspension more than 30 minutes old; (5) performing tests at cold room temperature.

The Quantitative Kahn Test of Blood Serum

The quantitative Kahn test is made only on sera giving 4+ or 3+ reactions with the standard Kahn test. Its purpose is to determine the exact number of reaction units in a positive serum. Thus, a serum may give a 4+ reaction in the routine Kahn test, but if diluted might become negative. Another serum may be able to withstand a dilution of many volumes and still react as a positive serum.

Physiologic salt solution is used as the diluent.

Prepare serum dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and higher if necessary, as follows:

Pipette 0.5 c.c. of 0.9% sodium chloride solution into each of six or more tubes.

Add 0.5 c.c. of inactivated serum to the first tube and mix.

Transfer 0.5 c.c. from the first to the second tube and mix.

Continue transferring and mixing from one tube to the next until all dilutions have been made. Allow the mixing pipette to remain in the last tube (so that further dilutions can be made if necessary).

Note: Serum dilutions should be used in the test soon after they have been prepared.

Prepare antigen suspension as previously described by mixing standard Kahn antigen with 0.9% sodium chloride solution, the quantity of saline according to the titer.

After the antigen suspension has stood at least 10 minutes but not more than 30 minutes, cover the mouth of the mixing vial containing the suspension and shake gently to obtain a smooth suspension of antigen particles.

Pipette 0.0125 c.c. of antigen suspension to the bottom of each of 6 or more numbered Kahn tubes.

Add 0.15 c.c. of the 1:64 dilution of serum to the antigen suspension in tube 6.

Add 0.15 c.c. of the 1:32 dilution of serum to the antigen suspension in tube 5.

Continue adding 0.15 c.c. of decreasing dilutions of serum to tubes 4, 3, 2, and 1, respectively.

Shake the rack by hand for 10 seconds and then allow it to stand for 3 to 7 minutes at room temperature.

Shake in a mechanical shaker for 3 minutes.

Add 0.5 c.c. of 0.9% sodium chloride solution to each tube.

Note: Add the saline to one rack and complete the reading before adding saline to another rack.

Shake the rack by hand a few seconds.

Read each tube within 2 minutes after the addition of the 0.9% saline solution to those in the rack.

Note the titration end point; that is, the highest dilution of serum in which 4+, 3+, or 2+ reactions are observed.

Compute the quantitative titer by applying the formula $S = 4D$ where S is the potency of the serum in terms of Kahn units, and D is the highest dilution demonstrating definite flocculation.

Examples:

(a) Highest dilution demonstrating definite flocculation is 1:64. $S = 4 \times 64$, or 256 Kahn units.

(b) Highest dilution demonstrating definite flocculation is 1:16. $S = 4 \times 16$, or 64 Kahn units.

Report results both in terms of Kahn units and the highest serum dilution in which a 4+, 3+, or 2+ reaction is observed:

(a) 256 Kahn units (1:64 dilution).

(b) 64 Kahn units (1:16 dilution).

Sera giving 3+, 2+, or 1+ reactions in the Kahn qualitative test may be reported as 3, 2, or 1 Kahn units, respectively, in the quantitative test if serum dilutions are nonreactive.

False Precipitate in the Kahn Test

Occasionally there is a false precipitate in the Kahn test. The late J. C. Willette, former Chief of Laboratories, St. Louis Health Division, in a personal communication stated that there were many factors that cause this trouble, among others the following: "It was discovered that we were using an ordinary commercial grade of nonabsorbent cotton to plug all of our glassware—that in the process of hot air sterilization the fat contained in the cotton plugs was vaporized; as the glassware cooled the fat condensed on the surface forming an almost imperceptible fatty film which was readily washed off by aqueous diluents forming the troublesome flocculent precipitate in our Kahn and agglutination tests.

"This difficulty, we found, had been encountered by others, and has resulted in the trade furnishing the laboratory worker with a fat-free nonabsorbent cotton, which satisfactorily disposes of this factor as a cause of false precipitates in flocculation and agglutination tests."

THE SPINAL FLUID KAHN PRECIPITATION TEST

Standard Qualitative Kahn Test of Spinal Fluid

Reagent.—

Saturated Ammonium Sulphate Solution.—

To 500 gm. of ammonium sulphate, c.p., reagent grade, add 500 c.c. doubly distilled water, in a clean 3 liter Pyrex flask.

Boil until solution becomes clear.

Allow to cool to room temperature.

Filter and store in glass-stoppered bottles at room temperature.

Technic.—

Spinal fluid should be clear. Centrifuge to remove cells or foreign matter, and decant the clear supernatant fluid.

Place 1.5 c.c. spinal fluid in a Kahn tube.

Add 1.5 c.c. saturated solution of ammonium sulphate. Mix well.

Place in a water bath at 56° C. for 15 minutes to precipitate the globulin.

Centrifuge at 2,000 r.p.m. for 15 minutes.

Pour off the supernatant fluid by inverting the tube, removing the last drop by means of filter paper. Drain 10 minutes on the filter paper.

Add to the residue in the centrifuge tube 0.15 c.c. saline, placing the pipette to the bottom of the tube just above the precipitate, and mix.

The spinal fluid is now ready for the test.

Dilute the antigen according to its titer. Allow to stand 10 minutes.

Place 0.01 c.c. antigen suspension in the bottom of a Kahn tube.

Add 0.15 c.c. concentrated spinal fluid.

Shake the rack by hand for 10 seconds, then in the Kahn shaker for 4 minutes.

Add 0.5 c.c. saline to each tube, shake by hand, and read.

Reading Results.—

Use Table 214 for reporting results.

TABLE 214.—REPORTING KAHN STANDARD QUALITATIVE TESTS WITH SPINAL FLUID

READING	REPORT
4+	Reactive, 4+
3+	Reactive, 3+
2+	Reactive, 2+
1+	Weakly reactive, 1+
±	Nonreactive
–	Nonreactive

Kahn Standard Quantitative Test With Spinal Fluid

Perform quantitative tests on spinal fluids which give reactive results in the qualitative test.

Whole spinal fluid is considered 1:10 dilution, since the qualitative test is made on spinal fluid globulin concentrated 10 times.

DILUTION	SALINE (C.C.)	SPINAL FLUID (C.C.)
1:10	None	
1:15	0.1	0.2
1:20	0.2	0.2
1:30	0.2	0.1
1:40	0.3	0.1

Place 0.01 c.c. antigen directly in the bottom of a Kahn tube. Use 1 tube for each dilution of spinal fluid being tested.

Add 0.15 c.c. diluted spinal to its respective tube, beginning with the highest dilution.

Shake the rack by hand for 10 seconds and then in a Kahn shaker for 4 minutes.

Add 0.5 c.c. saline to each tube, mix, and read immediately.

Note the highest dilution of spinal fluid producing a reactive result, 4+, 3+, or 2+.

Calculate the Kahn units according to the formula S = 4D,

where *S* is the potency of the spinal fluid in terms of Kahn units,

and *D* is the highest dilution in which a 4+, 3+, or 2+ reaction is observed.

Example:

(a) Spinal fluid reactive at 1:10 dilution (designated) = 10 × 4 = 40 Kahn units.

(b) Spinal fluid reactive at 1:40 dilution (designated) = 40 × 4 = 160 Kahn units.

Retest spinal fluids that produce only nonreactive results in the designated dilutions in the following manner:

Prepare a spinal fluid globulin concentrate, page 2078.

Add sufficient saline to make a 1:5 dilution, 0.15 c.c. of globulin solution plus 0.6 c.c. saline.

Perform a 1-tube test as described for testing spinal fluid dilutions.

If this gives a reactive result, the quantitative titer is 20 Kahn units; if it gives a nonreactive result, the titer is equivalent to the reading obtained with the undiluted globulin concentrate.

KLINE MICROSCOPIC SLIDE PRECIPITATION TESTS FOR THE DIAGNOSIS OF SYPHILIS

Patient's Serum

Prepare sera as for the Wassermann test. Sera must contain no red blood cells and no foreign particles. Heat the sera at 56° C. in an inactivator for 30 minutes.

Instrument for Making Paraffin Rings

This is essentially the instrument proposed by Green. Wind a piece of soft wire (No. 28) twice tightly about a 15 mm. diameter test tube, forming a double loop and leaving a double shaft about an inch in length. Twist the two shafts together to within a quarter of an inch of the free end. Remove the looped wire from the test tube. Use No. 12 linen thread to wind the wire. Begin at the free end of the shaft after fastening the thread there by a single twist of the free ends. Make three long turns to reach the loop, then wind tightly with the thread. Continue the winding up the shaft to the free end, and fasten it there between the two ends of the wire by twisting them. Bend the loop at right angles to the shaft. Reshape by working against the bottom of the test tube. Insert the shaft into the handle of a teasing needle or into straight hemostatic forceps. This loop is used to make the proper size rings for diagnostic and exclusion heated serum tests, diagnostic unheated serum, and finger blood tests.

To make paraffin rings, dip the instrument into smoking paraffin, at about 120° C., drain quickly at one point, and transfer the remainder to a glass slide.

Glassware

Slides are the standard Kline slides, 2 × 3 inches. These must be clean and grease-free.

Mount 12 paraffin rings, each with an inside diameter of 14 mm.

For spinal fluid tests, prepare double slides.

Pipettes are 0.2 c.c. graduated in 0.01 c.c. to the tip.

Centrifuge tubes, round-bottomed, 3 × 1 inch.

Bottles, round, glass-stoppered, 30 c.c. capacity.

Syringe, glass, hypodermic, 1 or 2 c.c. capacity.

Reagents

Antigen.—

Antigen for the Kline standard tests may be procured from LaMotte Chemical Products Co., Towson 4, Md. It is composed of cardiolipin, 0.2%, and purified lecithin, 1.6 to 2.6%, in absolute alcohol. It must be standardized by comparison with an antigen of standard reactivity. Store in a refrigerator at 6° to 10° C.

Cholesterol Solution.—

Dissolve 1 gm. of cholesterol (Pfanstiehl, ash-free, precipitated from alcohol) in 100 c.c. of absolute alcohol.

Store in a glass-stoppered bottle at room temperature.

Distilled water.—

This should have a reaction of pH 6.0, with a minimum number of positive ions or other electrolytes.

Saline.—

This is an 0.85% solution of sodium chloride in distilled water. It should preferably be made on the day of use, but may be satisfactory for as long as a week if kept in a clean glass-stoppered bottle.

Preparation of Antigen Emulsion.—

Pipette 0.85 c.c. of distilled water to the bottom of a 30 c.c. glass-stoppered bottle.

Add 1 c.c. of 1% cholesterol solution by allowing the cholesterol to drop slowly from a pipette while the bottle, held at an angle, is vigorously and continuously rotated on a flat surface.

Rotate for an additional 20 seconds.

Add 0.1 c.c. of antigen against the side of the neck of the bottle from a 0.2 c.c. pipette, avoiding the ground glass area.

Place the stopper in the bottle and shake vigorously for 1 minute, throwing the fluid from the bottom to the stopper and back.

Add 2.45 c.c. of 0.85% saline rapidly to the bottle and shake less vigorously for 30 seconds.

The emulsion is ready for use. If refrigerated it may be used for 48 hours. Double quantities of the antigen emulsion may be prepared in 30 c.c. bottles.

Preliminary Kline Test With Serum

Check the delivery of the hypodermic needle and make adjustments so that approximately 125 drops are obtained from each c.c. of antigen emulsion (0.008 c.c. per drop).

Complete tests with control sera of graded reactivity.

Reactions with control sera should reproduce the reactivity pattern. The nonreactive serum should show complete dispersion of antigen particles and the optimum number of particles per microscopic field.

Kline Standard Qualitative Test With Serum

Place 0.05 c.c. of heated serum in a paraffin ring on a glass slide.

Add 1 drop (0.008 c.c.) of antigen emulsion to each serum.

Rotate slides on a rotating machine at 180 r.p.m. for 4 minutes.

If tests are made in a hot, dry climate, cover with a box lid containing a moistened blotter, during rotation, to prevent excess evaporation.

Examine microscopically, using 100× magnification.

Report as follows:

(a) *Typical Reactions.—*

Nonreactive	Antigen particles dispersed, no clumping
Weakly reactive (± or 1+)	Antigen particles in small but definite clumps
Reactive (2+, 3+, or 4+)	Antigen particles in medium-sized or large clumps

(b) *Atypical Reactions.—*

Atypical reactions are characterized by irregular feathery clumping in which smaller clumps predominate. Atypical reacting sera should be retested in dilutions of 1:2 to 1:64. A reactive report is given if a reactive result is obtained with 1 or more serum dilutions.

Kline Standard Quantitative Test With Serum

Add 0.5 c.c. of saline to each of 6 or more tubes.

Add 0.5 c.c. of heated serum to the first tube and mix.

Transfer 0.5 c.c. from the first to the second tube and mix.

Continue in this manner until the last tube contains 1.0 c.c.

Pipette 0.05 c.c. of undiluted serum and of each serum dilution into separate paraffin rings on a glass slide.

Add 1 drop of antigen emulsion (0.008 c.c.) to each serum dilution.

Rotate slide at 180 r.p.m. for 4 minutes.

Read and record reactions as above.

Report results in terms of the highest dilution which produces a reactive result (4+, 3+, or 2+).

Example:

UNDILUTED SERUM	SERUM DILUTIONS						
1:1	1:2	1:4	1:8	1:16	1:32	1:64	
4	4	3	1	–	–	–	Reactive, 1:4 dilution or 4 dils
4	4	4	4	2	–	–	Reactive, 1:16 dilution or 16 dils
4	4	4	4	4	2	–	Reactive, 1:32 dilution or 32 dils

Kline Tests With Spinal Fluid

Preparation of Spinal Fluid.—

Spinal fluids turbid with exudate, blood, or bacteria, or containing injected substances including horse serum, are unsatisfactory for testing. Centrifuge spinal fluids with slight turbidity or few particles at 2,000 r.p.m. for 5 minutes and withdraw or decant the clear fluid.

Place the required number of test tubes, 6 × ⅝ inch, each containing 5 c.c. of Benedict's solution (1926), in a beaker (Pyrex). Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Place the tubes in a rack. After making certain no copper reduction has occurred in any of the tubes, add to each tube, properly numbered, 0.5 c.c. of spinal fluid. Shake tubes individually vigorously for ten seconds.

Replace the tubes in the beaker. Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Replace the tubes in the rack, inspecting each immediately after removal from the beaker for precipitate indicating presence of sugar.

Spinal fluids giving a negative reaction for sugar in the above test are unsatisfactory for testing for syphilis. These are fluids that have been acted upon by bacteria either inside or outside of the body. In the former case of the various bacterial meningitides in which organisms and ferments of the exudate have acted upon the sugar, the fluids may contain substances giving positive or unsatisfactory reactions in various tests for syphilis. Bacterial contamination subsequent to withdrawal of spinal fluids from cases of syphilis causes a steady loss both of sugar and of the specific reacting substance if the fluids are kept at room temperature.

On the other hand, if spinal fluids containing sugar when withdrawn from the body are kept at low temperature (8° to 10° C.) they continue to give a positive reaction for sugar with the test described above for several weeks, and syphilitic spinal fluids under these conditions show no appreciable loss of specific reacting substance for at least a week.

Preparation of Double-Ring Slides.—

Use clean Kline slides, 3 × 2 inches.

Place steel mold* and two center disks on slide.

Fill spaces between disks and outer mold with hot paraffin mixture, containing 1 part of paraffin and 2 parts of petroleum jelly.

Remove mold and disks from slide after paraffin has cooled.

Preparation of Antigen Emulsion for Spinal Fluid.—

Pipette 0.6 c.c. of 1% cholesterol solution to the bottom of a 3 × 1 inch, round-bottomed test tube.

Add 0.4 c.c. of distilled water rapidly by removing the finger from the pipette and blowing in the last drop while vigorously rotating the tube on a flat surface.

Continue rotation of the tube for another 10 seconds.

Add 0.1 c.c. of antigen and rotate the tube vigorously for 1 minute.

Add 1.4 c.c. of saline and rotate the tube on a flat surface for 30 seconds.

Centrifuge at 1,100 r.p.m. for 5 minutes. This time and speed may vary. The sediment should be in such an amount that when resuspended in 0.6 c.c. of saline, 1 drop, or 0.008 c.c., of the emulsion in 0.3 c.c. of spinal fluid will contain the optimum number of antigen particles per microscopic field.

Decant the turbid supernatant fluid.

Add 0.6 c.c. of saline to the sediment and rotate the tube vigorously for 30 seconds to resuspend the particles uniformly.

Transfer the emulsion to a stoppered 13 × 100 mm. tube. If stored in a refrigerator, the emulsion may be used for 48 hours.

Preliminary Kline Tests With Spinal Fluid

Check the delivery of the hypodermic needle and adjust so that approximately 125 drops are obtained from each c.c. of antigen emulsion. Each drop is 0.008 c.c.

Complete tests with reactive spinal fluid and nonreactive spinal fluid controls as described under Kline standard qualitative test with spinal fluid.

Clumping of antigen particles will be observed in the reactive spinal fluid. The nonreactive spinal fluid should show complete dispersion of antigen particles and the optimum number of particles per microscopic field.

Kline Standard Qualitative Test With Spinal Fluid

Place the required number of double-ring slides in a holder.

Heat the spinal fluid at 56° C. for 5 minutes as previously directed.

Pipette 0.3 c.c. of warm spinal fluid into a ringed chamber. Include reactive and nonreactive controls.

Add 1 drop (0.008 c.c.) of antigen emulsion to the spinal fluid in each chamber.

Rotate the slides on a flat surface with moderate vigor for 30 seconds to distribute the antigen emulsion.

Move the slide holder back and forth rapidly, about 3 complete movements a second, a linear distance of one-fourth to one-half inch, for 8 minutes.

Examine reactions microscopically at 100× magnification and report observed results as follows:

Nonreactive------------------Antigen particles dispersed, no clumping
Weakly reactive (± or 1+)------Antigen particles in small but definite clumps
Reactive (4+, 3+, or 2+)--------Antigen particles in medium-sized or large clumps

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*This mold can be procured from LaMotte Chemical Products Co., Towson 4, Md.

Kline Standard Quantitative Test With Spinal Fluid

Prepare spinal fluid dilutions of 1:2, 1:4, 1:8, 1:16, etc., using nonreactive spinal fluid or 0.9% saline as the diluent.

Test undiluted spinal fluid and each spinal fluid dilution in the usual manner as for the qualitative test.

Report results in terms of the highest dilution which produces a reactive (4+, 3+, or 2+) result in the manner described for the quantitative test with serum.

THE MAZZINI MICROSCOPIC FLOCCULATION TEST FOR SYPHILIS[1]

Equipment.—

Rotating machine, adjustable to 100 to 180 r.p.m., circumscribing a circle ¾ inch in diameter on a horizontal plane.

Slide holder to accommodate 1 to 4 slides, 2 × 3 inches.

13 and 21 gauge hypodermic needles with bevels removed.

Kline slides, 2 × 3 inches, with 10 concavities, 16 mm. in diameter by 3 mm. in depth.*

Kline slides with 3 concavities, one, 38 mm. in diameter, and two, 20 mm., for spinal fluid tests.*

Round bottles, glass-stoppered or screw-capped, 30 c.c. capacity.

Luer syringe, 1 or 2 c.c. capacity, or an observation tube No. 420 LST.*

Reagents.—

Antigen.—

Antigen for this test is an alcoholic solution containing 0.025% cardiolipin, approximately 0.2% lecithin, and 0.75% to 0.9% cholesterol, previously serologically standardized against an antigen of known reactivity.

Buffered Saline Solution.—

This solution must have a pH of 6.3 to 6.4 and a salt concentration of 10 gm. per liter.

Sodium chloride, c.p.	8.1 gm.
Primary potassium phosphate (KH_2PO_4)	0.2 gm.
Secondary sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$)	1.7 gm.
Doubly distilled water	1,000.0 c.c.
N/1 hydrochloric acid	3.2 c.c.
Formaldehyde, Merck reagent grade	1.0 c.c.

Dissolve and filter. Check the hydrogen ion concentration. The colorimetric method is sufficiently accurate. Keep in a glass-stoppered Pyrex bottle. This will remain unchanged indefinitely. If the solution should become contaminated with particles of dirt, cotton fibers, or other debris, filter the solution so that particles may not be transferred to the suspension and be misinterpreted as a positive reaction.

Serum.—

Prepare sera as for the Wassermann, Kahn, Kolmer tests, etc. (page 2031). Inactivate at 55 to 56° C. for 30 minutes.

Visible precipitates after heating must be removed by centrifugalization and decantation, so as not to interfere with serologic reactions.

Specimens containing an excessive amount of fat must be reported as unsatisfactory.

Retest weakly reacting sera which have been heated and thereafter kept at room temperature or in a refrigerator for several hours by withdrawing a new portion of the sera from the clots.

Hemolyzed sera may be tested unless they are excessively viscous after heating.

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*Procurable from Serological Reagents Co., Indianapolis, Ind.

[1] Mazzini, L. Y.: Ven. Dis. Inform. 23: 123, 1942; 1955 Manual, Serologic Tests for Syphilis, U. S. Department of Health, Education, and Welfare.

Preparation of Antigen Emulsion

Pipette 0.4 c.c. buffered saline to the bottom of a 30 c.c. bottle.

Measure 0.4 c.c. of cholesterolized antigen, reading from the bottom of the pipette, with a 1 c.c. pipette graduated to the tip.

Hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being blown directly and at once into the saline from the pipette held in the right hand.

Mix by drawing the suspension into the pipette and blowing out exactly 6 times, returning all of the emulsion left in the pipette into the last mixture.

Add 2.6 c.c. of buffered saline, cap the bottle, and shake from the bottom to the top and back *50 times in 15 seconds.*

Preliminary Testing of Antigen Emulsion

Check the delivery of the syringe or observation tube, fitted with a 21 gauge needle. When this is held in a vertical position, 0.01 c.c. of antigen suspension per drop should be delivered.

Test control sera of graded reactivity and buffered saline in the usual manner for the qualitative test. The results should reproduce the reactivity pattern previously established for these sera, and should show complete dispersion of the antigen particles in both nonreactive serum and buffered saline.

Mazzini Qualitative Test With Serum

Pipette 0.03 c.c. of each serum into a separate concavity.

Add 1 drop of antigen emulsion (0.01 c.c.) to each.

Rotate slides on the rotating machine for 4 minutes. The speed should be 160 to 180 rotations per minute. If rotating by hand, circumscribe a 2 inch circle 120 times per minute.

Read each reaction microscopically using a low power objective (16 mm.) and a 6× ocular. Record and report all sera that are nonreactive (no clumping) and all that are reactive (4+).

Add 1 drop, about 0.05 c.c., of 0.9% saline from a syringe with a 13 gauge needle with cut-off bevel, to each test which gives a 1+, 2+, 3+, or atypical reaction.

Rotate for a second 4-minute period on a rotating machine at 100 to 120 r.p.m. or by hand.

Examine microscopically. Report as follows:

DESCRIPTION	READING	REPORT
No clumping or coarse reactions	–	Nonreactive
Very small clumps	1+	Weakly reactive
Small clumps	2+	Weakly reactive
Medium-sized clumps	3+	Reactive
Large clumps	4+	Reactive

Atypical Reactions:

Atypical reactions are characterized by irregular particle aggregates of various sizes in which the small clumps and free antigen particles predominate. Atypically reacting sera must be retested as for the quantitative test.

Mazzini Quantitative Test With Serum

Procedure for quantitative test with serum is the same as for the qualitative test except that each serum is tested in serial dilutions.

Place 6 test tubes in a rack. Place 0.5 c.c. buffered saline solution in each tube. Place 0.5 c.c. heated serum in tube 1. Mix contents thoroughly, and transfer 0.5 c.c. to tube 2, mix and transfer 0.5 c.c. to tube 3, and continue the process throughout the 6 tubes. Carry out tests on each dilution of serum, using 0.05 c.c. of each serum dilution and 1 drop of antigen suspension (used for the qualitative Mazzini test with serum) in each wax ring. Rotate for 4 minutes at 180 r.p.m.

Report results in terms of the highest dilution of serum giving a reactive result (4+ or 3+).

Example:

SERUM DILUTIONS						REPORT
1:2	1:4	1:8	1:16	1:32	1:64	
4	4	4	2	–	–	Reactive, 1:8 dilution or 8 dils.
4	3	1	–	–	–	Reactive, 1:4 dilution or 4 dils.
4	4	4	4	1	–	Reactive, 1:16 dilution or 16 dils.

Mazzini Qualitative Test With Spinal Fluid

Test spinal fluid as soon as possible after withdrawal. Do not use grossly contaminated fluids. The spinal fluid should not contain blood. Do not heat the spinal fluid.

Centrifuge the fresh spinal fluid at 2,000 r.p.m. for 5 minutes. Pour off the clear supernatant fluid into a clean tube.

Pipette 0.05 c.c. and 0.2 c.c. of spinal fluid, respectively, into each of the two 20 mm. chambers, and 0.5 c.c. of spinal fluid into the 38 mm. chamber of the special glass slide.

Include known reactive and nonreactive spinal fluids as controls.

Add 1 drop (0.01 c.c.) of antigen emulsion to each chamber.

Rotate slides on a mechanical rotator for 10 minutes at 100 to 120 r.p.m.

Add 2 drops of 0.9% saline, immediately, from a syringe using a 13 gauge needle with cut-off bevel, to the chamber containing 0.05 c.c. of spinal fluid.

Rotate for another 20 minutes at 80 to 100 r.p.m.

Record results as for the serum tests. Report the results of the strongest reactions regardless of which of the three quantities it might be.

Example:

SPINAL FLUID QUANTITY			REPORT
0.05 c.c.	0.2 c.c.	0.5 c.c.	
1+	2+	4+	Reactive
–	1+	3+	Reactive
4+	2+	2+	Reactive
2+	4+	3+	Reactive
–	1+	2+	Weakly reactive
–	–	1+	Weakly reactive

Mazzini Quantitative Test With Spinal Fluid

Prepare serial dilutions of spinal fluid, which has been shown to be strongly reactive, in buffered saline: 1:2, 1:4, 1:8, 1:16, etc.

Pipette 0.5 c.c. of each dilution into respective 38 mm. chambers.

Add 1 drop (0.01 c.c.) of antigen emulsion to each chamber containing spinal fluid.

Rotate the slides on a mechanical rotator for 10 minutes at 100 to 120 r.p.m.

Rerotate for 20 minutes longer, at 80 to 100 r.p.m.

Record results of each dilution.

Report in terms of the highest dilution giving a reactive result in the manner described for quantitative test with serum.

THE TREPONEMAL IMMOBILIZATION TEST IN THE DIAGNOSIS OF SYPHILIS*

Nelson and Mayer[1] succeeded in maintaining *Treponema pallidum* alive, motile, and virulent until 5 to 10 days on artificial medium largely free of tissue components. When suspensions of motile treponemes in this medium were added to normal sera along with complement, the treponemes remained alive and virulent up to 15 to 18 hours. In mixtures of syphilitic human and rabbit serum with complement, however, they became immobilized and were killed. This test is now known as the "*Treponema pallidum* immobilization (TPI) test." This immobilizing antibody has been found separate and distinct from the Wassermann antibody or reagin.

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*Antigen for this test is now available from Sylvana Chemical Company, 407 S. Jefferson St., Orange, N. J.

[1] J. Exper. Med. 89: 309, 1949.

Later information on this test is given by Miller and Slatkin.[1] This immobilizing antibody develops only in response to the treponematoses: syphilis, yaws, pinta, and bejel. This test is highly specific for the treponematoses. Certain limitations of the test are present: If treatment is given in the primary stage or early in the secondary stage of syphilis, the test may become negative with the passage of time. It is thought that the test remains positive for life if the patient is not treated until the latent phase of syphilis.

Miller's studies were directed toward five phases of the subject: (1) the place the treponemal immobilizing test should occupy as a guide to treatment; (2) the significance of the reactive or weakly reactive serologic test for syphilis; (3) the significance of the results of the treponemal immobilizing test on spinal fluid; (4) the significance of the reactive serologic test in a group of patients admitted to the hospital for other diseases; (5) the evidence for passive transfer of the immobilizing antibody from mother to baby and the length of persistence of the antibody in the baby.

Their conclusions were that (1) the treponemal immobilization test is a practical means of differentiating biologically false positive reactions from the true positive reactions indicative of syphilis; (2) the concept of significance of weakly reactive and reactive serologic tests in the absence of signs or history of signs of syphilis must be revised in the light of evidence gained from the results of the treponemal immobilizing test; (3) the treponemal immobilizing test and the spinal fluid are of value in the diagnosis of neurosyphilis in patients in whom the spinal fluid serologic test has become negative as a result of the passage of time or treatment; (4) in acute disseminated lupus erythematosus positive serologic test represents biologically false positive reactions in a high percentage of cases; (5) little evidence for the recurrence of biologically false positive reactions was found in sarcoidosis; (6) passive transfer of the immobilizing antibody across the placenta occurs in nonsyphilitic babies born of syphilitic mothers; (7) in passive transfer the immobilizing antibody persists up to six months, whereas reagin disappears before three months.

According to Public Health Service Publication No. 327, in a booklet entitled *Management of Venereal Disease,* in cases with doubtful diagnoses (of syphilis), "it is anticipated that there will be seen an increasingly large number of patients who have a positive or doubtful standard test for syphilis as the sole referring complaint. In this group, a *Treponema pallidum* immobilization test may be found helpful when considered in the light of a complete study of the patient."

Method*

The treponemal immobilization test in the diagnosis of syphilis, introduced by Nelson and Mayer in 1949,[2] is the first successful in vitro measurement of specific treponemal antibody in syphilis. This antibody has been shown to be distinct from syphilitic reagin. It is now believed that the mechanism of the reaction is based on the specific combination of antigen and antibody. Combination will take place in the absence of complement, but immobilization will not ensue until complement has been added. Loss of motility of the organism in the presence of syphilitic serum and complement is believed to indicate death of the organism.

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*Compiled chiefly from Chorpenning, Frank W., U. S. Armed Forces M. J. 4: 807, 1953.

[1] J. A. M. A. 154: 1241, 1954.

[2] J. Exper. Med. 89: 309, 1949.

The amount of complement fixed appears to be small. The reaction depends on the temperature and the concentrations of antigen, antibody, and complement.

The test is of particular value when dealing with a serum from a clinically negative patient, whose serum gives a positive serologic test with standard tests for syphilis. This might be due to biologically false positive reactions, or it may be from a latent syphilitic patient.

Medium.—

1. **Bovine Plasma, Fraction V:**

Dissolve 20 gm. of the Armour Co. powder
in 350 c.c. of 0.85% sodium chloride solution.

Add sufficient M/1 sodium hydroxide (about 4.5 c.c.) to adjust the reaction to pH 7.0.

Dilute to 400 c.c. with 0.85% sodium chloride solution.

Sterilize by Seitz filtration and store at 5° C. Renew every second week.

2. **Phosphate Buffer, pH 7.1:**

(a) Dissolve 7.1 gm. of disodium phosphate
in 500 c.c. of distilled water.

(b) Dissolve 10.21 gm. of monopotassium phosphate
in 500 c.c. of distilled water.

(c) Mix 120 c.c. of (a) with 30 c.c. of (b) and sterilize by Seitz filtration. Store at 5° C. Renew biweekly.

3. **Sodium Bicarbonate, 1.25% Solution:**

Dissolve 1.25 gm. of sodium bicarbonate in distilled water and dilute to 100 c.c. with distilled water.

Sterilize by filtration through a glass-sintered filter and store at 5° C. Renew biweekly.

4. **Sodium Chloride, 0.85% Solution:**

Dissolve 8.5 gm. sodium chloride in 1,000 c.c. distilled water. Sterilize and store as directed in No. 3.

5. **Sodium Thioglycollate, 1.5% Solution:**

Dissolve 1.5 gm. of thioglycollate (order freshly made powder from Baltimore Biological Laboratory) in enough distilled water to make 100 c.c. solution.

Sterilize by filtration through a glass-sintered filter.

Plug, close tube with paraffin, and store at –20° C. Renew biweekly.

6. **Cysteine Hydrochloride, 0.63% Solution:**

Dissolve 0.63 gm. of cysteine hydrochloride, Matheron†
in 100 c.c. of 0.85% sodium chloride solution.

Sterilize and store as in No. 5.

7. **Glutathione, 1.23% Solution:**

Dissolve 1.23 gm. of glutathione, Matheron†
in 100 c.c. of 0.85% sodium chloride solution.

Sterilize and store as in No. 5.

8. **Sodium Pyruvate, 1% Solution:**

Dissolve 1 gm. of sodium pyruvate, Nutritional Biochemicals,‡
in 100 c.c. of 0.85% sodium chloride solution.

Sterilize and store as in No. 5.

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†East Rutherford, N. J.

‡Cleveland 28, Ohio.

9. **Ultrafiltrate of Bovine Serum:**

Procure a batch of ultrafiltrate of bovine serum without indicator from the Microbiological Associates, P.O. Box 5970, Washington 14, D. C.

10. **Composition of the Medium:**

Mix under sterile precautions.

Bovine plasma solution	50 c.c.
0.85% sodium chloride solution	11.6 c.c.
Phosphate buffer	15.65 c.c.
Sodium bicarbonate solution	5.65 c.c.
Glutathione solution	3.15 c.c.
Cysteine solution	3.15 c.c.
Thioglycollate solution	3.0 c.c.
Pyruvate solution	1.55 c.c.
Bovine serum filtrate	6.25 c.c.

Keep in a deep freezing unit.

Treponemal Suspension.*—

Treponema pallidum (Nichols strain) is used.

Inoculate intratesticularly male rabbits of about 2.5 kilograms weight.

Maintain in a new group of rabbits by passage immediately after onset of a definite orchitis. After preliminary passages, the average incubation period can be made to approximate 8 days.

After the early orchitis (1 or 2 days after onset) has been diagnosed by palpation, sacrifice the rabbit by injecting air into the marginal ear vein, aseptically remove the testicular syphiloma from the scrotal sac, and slice the testicle by making eight transverse and one longitudinal sections.

Wash once in about 20 c.c. of chilled, sterile 0.85% saline solution.

Place the slices in a flask containing 30 c.c. of medium for each testicle under an atmosphere of 95% nitrogen and 5% carbon dioxide.

Gently agitate, mechanically, at 40 to 45 oscillations per minute, for 1 or 2 hours at a temperature of 35° C.

Obtain counts of the number of treponemas per c.c. in the suspension by dark-field examination after 1 hour, and again after 2 hours, if the first count is unsatisfactory.

Between 3 and 5 organisms per high dry field (or 5,000,000 per c.c.) is optimal. If more are obtained, dilute with medium.

Pour off the treponemal suspension.

Centrifuge for 10 minutes at 1,000 G to remove the gross debris.

Use the supernatant fluid for the test.

Chorpenning and associates[2] recommend the use of frozen syphilomatous testicles. This method would allow the setting up of a center from which branch or subordinate laboratories could be supplied.

The test proper must be set up under sterile precautions. Some authors recommend loose plugging of all tubes. Sera and complement must be cold before use. All examinations of the end results must be carried out within one hour after the tubes have been taken out of the nitrogen-carbon dioxide jars, to prevent death of the organisms under aerobic conditions.

Technic of the Test.—

Carry out the test in Kahn tubes.

Place 0.05 c.c. of inactivated (56° C., 30 min.) serum of the patient in a Kahn tube.

Add 0.05 c.c. of undiluted guinea pig serum (complement).

Add 0.4 c.c. of treponemal suspension.

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*Antigen for the TPI test is now available as TPCF and TPIA antigen from Sylvana Chemical Company,, 407 S. Jefferson St., Orange, N. J.

[2] Am. J. Syph. 36: 401, 1952.

The complement is best prepared by bleeding 10 to 12 guinea pigs and pooling their sera. It will keep for use in this test for about 1 month, if kept frozen in a freezer unit.

Controls.—

(1) Place in a Kahn tube
0.05 c.c. of serum to be tested
0.05 c.c. of inactivated guinea pig serum (heated in a water bath at 56° C. for 30 minutes)
0.4 c.c. of treponemal suspension

(2) 0.1 c.c. saline
0.4 c.c. treponemal suspension

(3) 0.05 c.c. of ultrafiltrate of bovine serum or nonsyphilitic human serum
0.05 c.c. of pooled guinea pig serum
0.4 c.c. of treponemal suspension

(4) 0.05 c.c. of ultrafiltrate of bovine serum or nonsyphilitic human serum
0.05 c.c. of inactivated pooled guinea pig serum (heated in a water bath at 56° C. for 30 minutes)
0.4 c.c. of treponemal suspension

Incubate the test and all controls in a 95% N and 5% CO_2 atmosphere at 35° C. for 18 hours.

Remove a portion of the test suspension by means of a platinum loop and prepare a wet mount for dark-field examination.

Determine the number of motile organisms in the control tube (1) and figure the per cent of motile organisms present.

Determine the per cent of motile organisms in the test.

Subtract the per cent of motile organisms in the test from the per cent of motile organisms in the control.

Multiply this by 100 and divide by the per cent of motile organisms in the control tube. This gives the per cent of treponemas specifically immobilized.

50% and more immobilization is considered a positive test.

20 to 49% is considered a doubtful reaction.

Below 20% is considered negative.

A check for the utilization of the complement is carried out by adding 0.1 c.c. of sensitized 5% sheep cells to each test tube after the reactions have been read and the results recorded. The sensitized sheep blood cells are prepared as in the Kolmer qualitative test, using 2 units of hemolysin.

Incubate the tubes at 37° C. for 30 minutes. The presence of at least 2 plus hemolysis signifies an adequate surplus of complement which prevents false negative reactions.

If quantitative results are desired, the per cent of organisms specifically immobilized with the various serum dilutions (at least 3) can be recorded on semilogarithmic graph paper, plotting the reciprocal of the dilution logarithmically and the per cent of organisms specifically immobilized linearly. The reciprocal of the dilution at which specific immobilization of 50% of the treponemas occurs (IT_{50}) is read as the titer after method of Nelson and Diesendruck.[3]

Precautions.—

All sera to be tested should be sterile and free from preservatives, including penicillin. Penicillinase may be used to neutralize the presence of penicillin.

If the orchitides have progressed too far in the animal hosts, the treponemas are partially or completely immobilized by complement alone. This can be prevented if diagnosis of an early orchitis is made, by carefully examining the inoculated animals daily, to prevent the possibility of sensitization of the organisms to antibodies produced by the animals.

Interpretation.—

Patients with late syphilis consistently give positive results.

A few negative and doubtful tests occur in the early stages of syphilis. A period of antibody formation is necessary before serologic tests become positive. The titer follows a rising curve during this period.

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[3] J. Immunol. **66**: 667, 1951.

This test is at least 99% specific. It gives few, if any, positive results with non-syphilitic sera.

The test is positive in only about 57% of primary syphilitic cases.

In secondary syphilis, greater than 90% of the patients give positive reactions.

The greatest value of the TPI test lies in the latent, late symptomatic stages of syphilis, or with patients whose sera give biologic false positive reactions with the standard tests for syphilis.

The titer of the TPI test is not necessarily a direct measure of the patient's immunity.

There is a significant cross-reactivity between *Treponema pallidum, T. pertenue,* and *T. cuniculi,* although the degree of reactivity using *T. pallidum* and *T. pertenue* antisera against *T. cuniculi* and vice versa was consistently lower than with homologous antiserum or in cross-reactions between *T. pallidum* and *T. pertenue.*

The TPI test must be performed carefully. Difficulties lie in the need for fresh syphilomatous rabbit testes as a source of motile treponemas, which may be surmounted by preservation of the testicular syphiloma by freezing; the need for air-conditioned rabbit rooms; constant danger of obtaining partially sensitized treponemas from testicular lesions; the degree of reproducibility of quantitative results. Development of antibody in the rabbit may be inhibited or delayed by pre-treating the animals with nitrogen mustard or by x-radiation just prior to infection.

At the time of writing this text, the author was privileged to witness the entire performance of this test under the direction of Dr. John F. Kent, Chief of Serology, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C., to whom he wishes to express his heartfelt thanks for the courtesies extended to him by Dr. Kent and his assistants, Miss Barbara Rosenau and Mr. Robert W. Sanders.

In a form used by the Army, it is directed that 10 c.c. of sterile serum or cerebrospinal fluid be forwarded to the laboratory performing the test. In this same pamphlet the following criteria for submission of treponemal immobilization tests are set forth:

The TPI test *is not* indicated, and *should not* be requested under the following conditions:

1. Where the diagnosis of syphilis has been definitely established on the basis of unequivocal clinical and anamnestic evidence. This requirement would be fulfilled under the following conditions:

   a. Where *Treponema pallidum* has been identified by dark-field examination of primary or secondary lesions.

   b. Where a diagnosis of secondary syphilis made on clinical evidence (rash, etc.) has been corroborated by repeated positive reactions in the standard cardiolipin microflocculation (CM) and cardiolipin complement fixation (CCF) tests of the Army and Air Force.

   c. Where a patient, repeatedly seropositive in one or both of the standard cardiolipin tests (CM and/or CCF), has been shown to have evidence of visceral syphilis, especially involvement of the cardiovascular or central nervous systems. Cases of inactive syphilis of the central nervous system (Dattner-Thomas type, i.e., CCF and colloidal gold-positive, cell count and total protein within normal limits) will fall in this category.

2. Where a patient is an infant born of a mother with positive serology, in which case the positive serum reaction may be due to passively transmitted antibody.

The TPI test is indicated, and a request should be initiated under the following conditions:

1. Where the patient has been *repeatedly* seropositive in one or both of standard STS* and there is neither clinical nor anamnestic evidence of a syphilitic infection. There should be no evidence of visceral involvement, i.e., cardiovascular disease or CNS syphilis. Seropositivity should have persisted for three months *without significant reduction in titer* as determined by repeated (three) cardiolipin microflocculation tests (STS). (During this period no antisyphilitic chemotherapy should have been administered. The TPI test should be requested after the last standard serologic test for syphilis.†)

2. In the occasional instances where the patient has been repeatedly seronegative in both of the standard cardiolipin tests (STS confirmed over a three-month period), but shows evidence of visceral syphilis, e.g., cardiovascular involvement.

The result of the initial TPI test should be confirmed one week later by submitting a second specimen (10 c.c.) of sterile serum.

Frozen Syphilomatous Rabbit Testes as a Source of Treponema Pallidum for the Immobilization Test for Syphilis

Anderson, Kent, and Sanders[1] detail a schedule for utilizing frozen rabbit testes containing *T. pallidum.* This study gives results obtained with an additional 185 testes, many frozen for periods considerably exceeding those obtained at the time of the publication of the preliminary report.[2] In other words, this report shows that frozen material may be used for carrying out this test instead of having to depend on freshly procured material.

Rabbits were young males of mixed breeds, ranging in weight from 3.5 to 4.5 kilograms. They were caged in a room maintained at 18° to 20° C. by air-conditioning. Each animal was inoculated with 5-7 × 10^7 treponemes of the Nichols strain. Inoculum was contained in 1.0 c.c. of a 1:10 dilution of normal rabbit serum in 0.85% sodium chloride solution and was administered intratesticularly, 0.25 c.c. aliquots being introduced at two sites on each testis. Acute orchitis developed, on the average, eleven days thereafter, whereupon the animal was sacrificed by air embolism, and the syphilomatous testes were enucleated aseptically.

The technique of preservation was essentially that given in the preliminary report.[2] The testis was incised with a special instrument similar to that described by Chorpenning and associates, then washed with 2 to 3 c.c. of sterile 0.85% sodium chloride solution, and transferred aseptically to a sterile 25 by 150 mm. Pyrex test tube. The tube was plugged with gauze-wrapped cotton, and the cotton plug surmounted with a sterile rubber stopper. Finally, the testis was frozen quickly in a slush of methyl cellosolve and solid carbon dioxide (–78° C.), and the tube placed immediately in a cabinet refrigerated with solid carbon dioxide; storage temperature varied within the range –55° to –45° C.

When selected for tests, a syphilomatous testis was thawed by partially immersing the tube for 5 to 10 minutes in a water bath at 35° C.; the rubber stopper was removed as a safety measure during this operation. The thawed testis was transferred aseptically to a flask containing the prescribed survival medium, the treponemes were eluted, and the resulting suspension evaluated according to standard procedure for the immobilization test.

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*Serologic tests for syphilis.

†Attention in this connection is invited to TB Med. **230**: 8, 1952.

[1] Am. J. Syph. **38**: 527, 1954.

[2] Chorpenning, F. W., Sanders, R. W., and Kent, J. F.: Am. J. Syph., Gonorr. & Ven. Dis. **36**: 401, 1952.

Requests for the Nichols strain treponeme are filled by air-mailing frozen syphilomatous rabbit testis in a special container* refrigerated with solid carbon dioxide. Shipments have been made by these workers in this manner to facilities in Germany, Cuba, California, Michigan, and New York, where inocula from the frozen material were used successfully to initiate the treponemal infection in rabbits.

In this manner, actively motile *T. pallidum,* suitable for use in the treponemal immobilization test for syphilis, may be obtained from syphilomatous rabbit testes stored in the frozen state for periods varying from one day to one and one-half years.

At the date of this text writing, the author believes that this TPI test of Nelson and Mayer is a useful complement fixation test, using the immobilization of the *T. pallidum* as the indicator. He wishes to express his opinion that it is quite a difficult test to perform and expensive to operate. He believes that with a proper set-up with experienced workers the test is a valuable one, especially in pointing out biologic false positive reactions. It is not yet applicable to the ordinary hospital clinical pathological laboratory.

THE WASSERMANN AND HECHT-GRADWOHL COMPLEMENT FIXATION TESTS FOR GONORRHEA AND TUBERCULOSIS

We advise the purchase of the gonococcus and tubercle bacillus antigens for these tests from a reliable biological house. The titer and dilution of the antigen for use in complement fixation tests will be marked on the container.

Dilute the antigen according to the dilution given on the label.

The reagents used in these tests are the same as are used in the tests for syphilis with the exception of the antigen.

The technic for each test is the same as the test for syphilis. The only difference is the substitution for syphilis antigen of gonococcus antigen in the test for gonorrhea, and tubercle bacillus antigen in the test for tuberculosis.

The antigen for the Hecht-Gradwohl test is half the strength of the Wassermann antigen.

The results are reported as either positive or negative, dependent on whether there is inhibition of hemolysis or complete hemolysis of the sheep cells.

THE WELTMANN SEROCOAGULATION REACTION FOR TUBERCULOSIS[1, 2]

Studies in the field of tuberculosis have been made along the lines of both specific and nonspecific reactions. The complement fixation test in tuberculosis is an example of the specific reaction; the sedimentation test and the Weltmann serum coagulation reaction are examples of the nonspecific reactions.

The Weltmann test was introduced in 1930,[3] at which time it was claimed that this test would indicate either an exudative or a fibrotic process.

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*Virus Shipping Container, Model VSC-2C, Hollinger Corporation, Arlington, Va.

[1] J. Lab. & Clin. Med. 23: 994, 1938.

[2] Sedlacek, B., Miller, Ed. G., and Medical Staff, Navajo Medical Center, Fort Defiance, Ariz.: Lab. Digest 6: 3, Aug., 1942.

[3] Weltmann, O.: Med. Klin. 26: 240, 1930.

Levinson and Klein,[4] in 1938, reported on the Weltmann serum coagulation test. In general, acute inflammatory and exudative conditions give a shortened coagulation band or a "shift to the left." In chronic diseases, characterized by fibrotic changes, in the healing stage of acute infection, and in parenchymal liver damage, the coagulation band tends to be lengthened, or results in the "shift to the right." The coagulation band does not always parallel the sedimentation rate.[5] In tuberculosis the Weltmann coagulation test is of importance because it can be used as a guide in the course of and in the prognosis of the disease. It reflects tissue changes that occur in the body. Exudative changes in tuberculosis are manifested by a shortening of the coagulation band, while fibrotic changes are accompanied by a prolonged coagulation band. When exudative and fibrotic changes occur in the lung at the same time, as happens frequently in tuberculosis, the Weltmann reaction may not be of great assistance. The two divergent processes occurring at the same time result in the coagulation band's striking the normal zone. In miliary tuberculosis, peritoneal tuberculosis, and in meningeal tuberculosis, the Weltmann reaction gives a shortening of the coagulation band in the exudative zone. Isolated bone tuberculosis is accompanied by The Weltmann test has been applied not only to tuberculosis in the minimal, moderately advanced, and far advanced stages, but also to the complications that occur in pulmonary disease. It has also been employed and compared with the sedimentation rate in several examples of various types of tuberculosis over a prolonged period of time. The results are given in the text. Although unable at the present to explain the mechanism of the Weltmann serum coagulation reaction, it is believed that this test is of importance in the diagnosis and prognosis of disease, particularly in tuberculosis.

In normal pregnancy, the sedimentation rate is accelerated, while the Weltmann test gives a normal band.

The sedimentation rate varies according to the temperature of the room, and in normal individuals it may fluctuate within a 24-hour period, while the Weltmann test is unaffected by atmospheric conditions.

In patients receiving artificially induced fever, the coagulation band is normal, but it shows a shift to the left in febrile conditions. This indicates inflammatory changes such as exudation occurring in pulmonary tuberculosis, pleural effusion, or suppurative processes. If a normal band or a shift to the right is obtained in these cases, exudative processes must be ruled out. In the absence of inflammatory or exudative processes, the Weltmann test shows no deviation,[6] and therefore it is a means of determining quantitatively the degree of pathologic changes.

In artificial pneumothorax or bilateral pneumothorax, the coagulation band shows a shift to the right, paralleling clinical improvement of the patient.

Hartwell[7] used the Weltmann reaction in the management of patients with myocardial infarction. Long after the temperature, white count, and sedimentation rate had returned to normal, the Weltmann reaction gave incontrovertible proof that the area of infarcted muscle was not yet healed and that the patient should remain quiet for a while longer.

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[4] Am. Rev. Tuberc. 37: 200, 1938.

[5] Loc. cit.

[6] Sedlacek, B., and Miller, E. G.: Lab. Digest 6: 3, 1942.

[7] Hartwell, Alfred S.: Hawaii M. J. 4: 136, 1945.

The reaction is not affected by plasma proteins, hydrogen ion concentration, electrolytes, and bilirubin. Blood calcium level does affect the band slightly.

Technic:

From a stock solution of 10 per cent calcium chloride ($CaCl_2.6H_2O$), ten dilutions consisting of 0.1 per cent, 0.09 per cent, 0.08 per cent, 0.07 per cent, 0.06 per cent, 0.05 per cent, 0.04 per cent, 0.03 per cent, 0.02 per cent, and 0.01 per cent are made. These dilutions are numbered from one to ten, beginning with the strongest concentration. Ten small test tubes, such as are used in the Wassermann reaction, are placed in a metal rack and numbered in order from 1 to 10. Into each tube is pipetted 5 c.c. of the similarly numbered calcium chloride solution and 0.1 c.c. of unhemolyzed serum. The tubes are then shaken so that the contents are well mixed and placed in a boiling water-bath for fifteen minutes. They are then removed and the reaction can be immediately determined. The contents of the tubes may be clear, faintly opalescent, turbid, or there may be a flocculation. There is usually a sharp and easily noted difference between flocculation and turbidity.

The number of tubes in which coagulation occurs is designated by Weltmann as the coagulation band (C.B. of that particular serum). If there is a very slight or doubtful flocculation in one tube the reaction is interpreted as being intermediate between that tube and the one before it. In normal serum the first 6 tubes usually show flocculation. Sometimes there is a slight or doubtful flocculation in the seventh tube. The normal coagulation band is, therefore, 6 to 6½, and is remarkably constant. This means that normal human serum diluted 50 times with calcium chloride solution and heated in a boiling water bath for fifteen minutes will not coagulate nor flocculate if the concentration of the calcium chloride is less than 0.04 per cent. If the coagulation band is less than 6, Weltmann speaks of the reaction as showing a shift to the left, whereas, if the coagulation band is 7, or greater, the reaction indicates a shift to the right.

A **numerical system** of reporting by figures instead of by a graph has been worked out by McCune.[2]

Key for Interpretation of Weltmann Reaction (McCune)

Complete flocculation—100%; partial flocculation—75%; turbid but no flocculation—50%; opalescent—25%; clear—0. The coagulation number is the sum of the individual tubes expressed in terms of percentage.

Below 650 to 0..........Exudative
700 to 650..............Slightly exudative
From 700 to 775.........Normal
From 775 to 825.........Slightly fibrotic
Above 825...............Fibrotic

For example, the first two tubes show complete flocculation.	2 × 100 = 200
The third and fourth tubes show partial flocculation.	2 × 75 = 150
The fifth and sixth tubes show turbidity.	2 × 50 = 100
The seventh and eighth tubes are opalescent.	2 × 25 = 50
The ninth and tenth tubes remain clear.	2 × 0 = 0
Coagulation number	500,

which indicates an *exudative* process.

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[2] Loc. cit.

An example of a *fibrotic* process is:

The first seven tubes show complete flocculation.	7 × 100 = 700
The eighth tube is only partially flocculated.	1 × 75 = 75
The ninth tube is turbid.	1 × 50 = 50
The tenth tube is opalescent.	1 × 25 = 25
Coagulation number	850

Exudative Conditions or Shift to the Left With a Total Coagulation Number Below 700.—Lobar pneumonia shows extreme shift to the left, almost to zero, during the stage of consolidation, or a total of about 450. In acute rheumatic fever and peritoneal tuberculosis, the total is not over 550. Nephrotic syndrome, exudative pulmonary tuberculosis, meningitis, miliary tuberculosis, peritoneal tuberculosis, appendiceal abscess, and exudative lobar pneumonia may give a total as low as zero. Pulmonary tuberculosis gives a total of about 500 or lower after the patient has received artificial pneumothorax or bilateral pneumothorax for some time where x-ray of the lung shows improvement; this may be accompanied by complication in the pleural cavity. Pyelitis gives a total of about 525.

Fibrotic Conditions or a Shift to the Right With a Total Coagulation Number Over 775.—Cirrhosis of the liver, fibrotic tuberculosis, septic states, endocarditis lenta, peritoneal carcinoma will give an extreme right shift. Pulmonary tuberculosis, when the shift is to the right or over 775, is paralleled by clinical improvement of the patient. Tuberculosis of the bone, chronic diseases characterized by fibrotic changes, acute infections, parenchymal liver damage in the healing stage, and trachoma will give a right shift.

Complement Fixation Test for Amebiasis is discussed on pages 1831 and 1835.

Complement Fixation Tests for Viruses and Rickettsial Diseases are discussed on page 1742.

The **Cold Hemagglutination Test in the Diagnosis of Primary Atypical Pneumonia** is discussed on page 1757.

The **Hemagglutinin Inhibition Test** is described on page 1754.

CHAPTER XIV

TISSUE CUTTING AND STAINING

Preparation of Tissues

There are two methods commonly used in the preparation of tissues: (1) the frozen section; and (2) the embedding method either in paraffin or celloidin.

Tissue Fixation

The term "tissue fixation" means the preservation of the normal structure of a tissue as closely as possible to its condition during life. It is necessary, therefore, to place tissues intended for microscopic examination in a fixative solution as soon as possible after they are removed from the body and cut off from the normal circulation. The ideal fixative would be one that preserves tissue exactly as it appeared during life but this is next to impossible to accomplish. The most commonly used fixative is a solution of formaldehyde. Formaldehyde is a gas, soluble in water to the extent of 40 per cent by weight. It has the advantages of penetration and fixation with great speed, thus enabling one to cut and stain frozen sections as well as paraffin sections. Its best strength is a 10 per cent solution, which is prepared by taking one volume of commercial or 40 per cent formeldahyde to nine volumes of tap water or saline. In order to prevent formaldehyde from developing formic acid, which is injurious to the tissue, the stock solution of formaldehyde should be neutralized by addition of 5 gm. of calcium carbonate to a gallon of fluid. When specimens are to be kept for a long time in this solution, place a few marble chips in the container.

Other fixatives are used, depending upon the type of pathologic lesion which is to be studied. Zenker's solution is one of the most reliable types of fixative since it preserves nuclear structure, bacteria, and fibrils of all kinds. Formula for this solution is found on page 2115. Make a stock solution of Zenker's fluid but do not add the acetic acid until immediately before using. Tissues are fixed from 12 to 16 hours in this solution, then washed overnight under tap water to remove all excess potassium bichromate, and transferred to 70 per cent alcohol.

Stowell[1] has made a distinct contribution to the effect on tissue volume of fixation, dehydration, and embedding. He found that all fixatives showed specific differences. Susa and Bouin fixatives gave the best general results, and neutral Formalin mixtures, especially pyridine-Formalin, the poorest. Isotonic saline was found superior to distilled water as a Formalin diluent, reducing tissue swelling during Formalin fixation. Reagents producing marked decreases in tissue volume render such tissues less susceptible to shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may exceed that produced by fixation alone. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage, with hot nitrocellulose less, and with cold nitrocellulose the least shrinkage.

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[1] Stain Technology 16: 2, 1941.

Alcohol is also useful as a fixative because it hardens and dehydrates tissue at the same time. Tissues kept in alcohol, however, cannot be immediately used for frozen section work. There is also a tendency on the part of alcohol to produce too great hardness in tissue, rendering it difficult for sectioning purposes. It does not penetrate as well as formaldehyde so the tissues must be cut in very small pieces.

In general, all tissue for fixation must be of a size sufficient for penetration. The volume of the fixative solution must be at least twenty-five times the volume of the tissue. It is therefore obvious that the smaller the tissue, the smaller the receptacle need be to get this 25:1 ratio.

Microtomes

The instrument used for cutting thin sections of tissue or other material for preparation for microscopic examination is called a *microtome*. These instruments are designed to cut material extremely thin, from 1 to 60 microns, and may or may not be provided with mechanical feeding mechanism. There are various types of microtomes:

Frozen sections are made with a microtome specially designed for this purpose known as an automatic microtome with a swinging carriage. The peculiar motion of the knife makes this microtome especially desirable for cutting **celloidin sections**, when equipped with freezing attachment. It is not adapted for paraffin work. The extreme ends of the knife rest in the carrier, and, as the lever handle moves the swinging arms, the knife describes a flattened curve, corresponding to the double movement of free-hand sectioning. By this manner of holding the knife by arms which are not parallel, the entire length of its cutting edge is utilized, insuring uniform wear and permitting the cutting of very large sections. With this microtome use a knife about 110 mm. long. The automatic feed mechanism consists of an accurately cut ratchet wheel, keyed to the vertical feed screw, in the teeth of which a hardened steel pawl engages. By means of a lever extending to the graduated scale on the front of the main frame base, this pawl may be thrown out of action or it may be set to cut sections of any desired thickness. Each division of the graduated scale marks 5 microns. Better results are obtained in making frozen sections if the automatic feeding mechanism is detached and the microtome operated by hand (see page 2103).

Possibly the best type of microtome for **paraffin work** is known as the Minot simplified rotary microtome, with universal ball and socket specimen clamp, designed in accordance with the wishes of the late Dr. Charles S. Minot, of Harvard University, to provide an instrument having the efficiency of the more elaborate rotary microtomes, but of simpler construction. The feeding apparatus operates automatically and may be adjusted to cut any thickness from 2 to 26 microns at intervals of 2 microns. The object holder consists of a disk 25 mm. in diameter which can be oriented to any angle and which moves on a vertical slide actuated by a crank operated by a heavy balance drive wheel. The latter is grooved for belt drive. The knife block consists of a heavy iron casting which is attached to the base and holds the knife in a fixed position, adjustable to and from the object and from side to side to permit the use of nearly the entire cutting edge. It may also be tilted in its support to set the angle of the cutting edge as desired. With this microtome use a plain knife 125 mm. long.

Another microtome which is intended for more accurate and more careful work is the Spencer precision rotary microtome. An inclined plane feed, which is independent of the up-and-down movement on the perpendicular support, overcomes the inaccuracies inherent in other rotary microtomes. The provision of an accurate and reliable feed mechanism, combined with the utmost rigidity of parts, makes this instrument very precise and accurate for laboratory or research work. Sections may be cut of any definite thickness, each section having the same thickness.

The feed mechanism is composed of a rigid bearing, on which the feed block is moved by an accurate screw, cut with two threads to the millimeter; it is revolved by a ratchet feed wheel with 250 teeth; each tooth represents, therefore, a movement forward of the object of one micron. The feed is so arranged that it can be set for sections of any thickness, from 1 micron to 60 microns, by turning the knurled button at the back of the case until the number representing the desired thickness appears opposite the indicator at the small opening in the side of the case near the balance wheel. The total excursion of the feed is 37 mm., allowing a sufficient range for cutting a complete series of a very large object without the necessity of a break due to resetting the knife and feed mechanism.

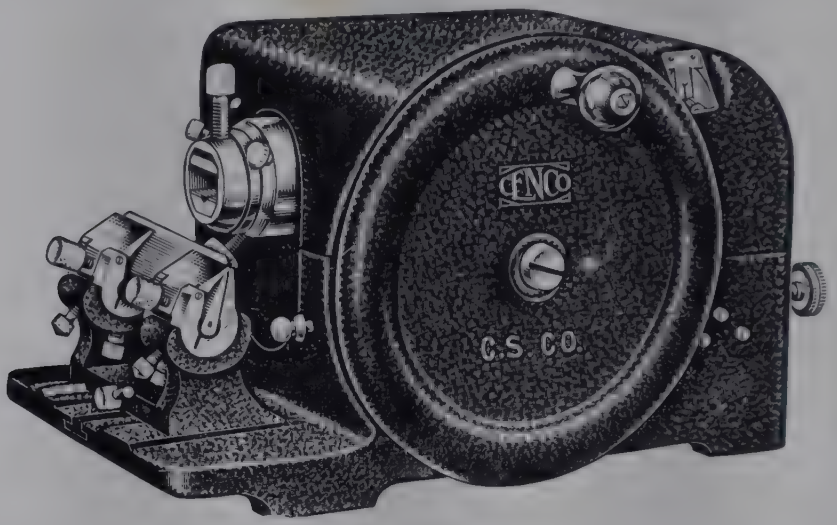

Fig. 606.—Minot simplified rotary microtome. (Courtesy Central Scientific Co.)

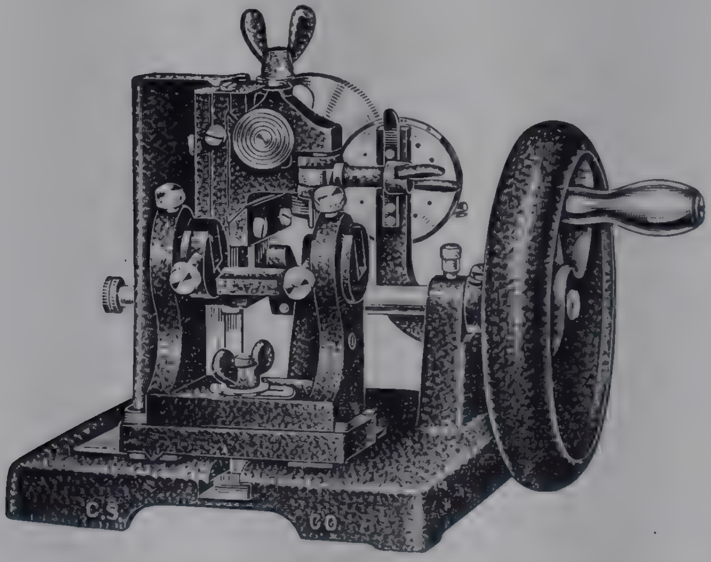

Fig. 607.—Spencer precision rotary microtome. (Courtesy Central Scientific Co.)

The up-and-down stroke of the object clamp is 50 mm. which permits the cutting of very large sections and gives sufficient stroke for celloidin cutting. The clamp may be held at its upper limit for orienting or trimming the block by pushing in a pin. The object may be rotated easily.

The knife is fastened by two clamps, each of which clamps the knife at the back and along 1¼ inches of the edge as well. It may be turned to any desired angle, and the clamps may also be moved toward one another to bring them as near to the ribbon as desired to gain additional rigidity. When the clamps are thus drawn together, the knife may be moved so that practically all of the cutting edge can be used before the necessity of resharpening. The adjustment screws provide for knives of different widths. The whole knife support is adjustable to and from the object, and is very easily and conveniently clamped in any location by a lever connected with an excentric cam.

The whole of the feeding mechanism is covered, protecting the wearing parts from dust. The top of the case is hinged to the lower part, permitting it to be easily opened. The balance wheel is grooved, so that the instrument may be run by a motor.

When desired, the microtome is provided with an adjustable knife support which is especially adapted to celloidin sections. The knife holder of this support is adjustable from a horizontal position through 55° upward.

Another type of microtome, which is very essential for celloidin embedded specimens, is a sliding microtome. This microtome is operated in much the same manner as the paraffin microtomes already described. The essential difference in this type of instrument is the fact that the knife moves against the object. The angle of the knife to the instrument is also different. In addition to this, in using this instrument, the object and the knife must be constantly wetted down with alcohol.

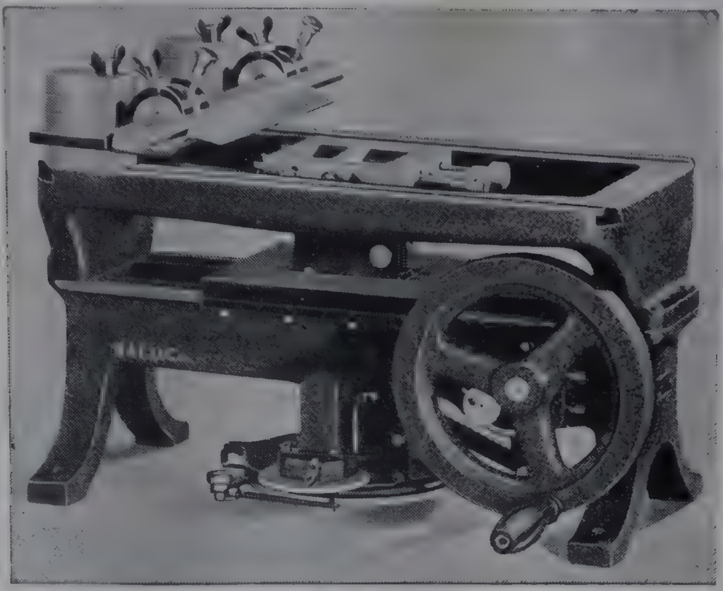

Fig. 608.—Sliding microtome. Can be used for both paraffin and frozen section technics. (Courtesy Bausch & Lomb Optical Co., Rochester, N. Y.)

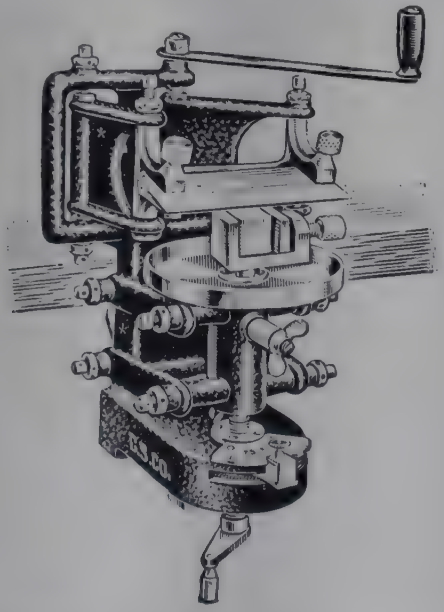

Fig. 609.—Spencer freezing microtome. Alternate method but not as good as sliding microtome method. (Courtesy Central Scientific Co.)

To Sharpen a Microtome Blade.[1, 2]—

Prepare the microtome knife for honing by screwing the handle into the threads provided at one end of the knife, then slide the sharpening back onto the knife. This sharpening back raises the knife and establishes the correct angle of the cutting facets. Take care to put the proper back onto the knife. Each knife has its own sharpening back; these backs should not be interchanged between knives.

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[1] The Micro Sharp Company, Box 363, Niagara Falls, New York, and Box 456, Toronto, Ontario, Canada, does excellent work in sharpening microtome blades. The author recommends this company to those who do not sharpen their own blades.

[2] For much of the material on the care and use of micotome we are indebted to Dr. O. W. Richards, Research Biologists, of American Optical Co., Scientific Instrument Division; this material is contained in a pamphlet by Dr. Richards, "The Effective Use and Proper Care of the Microtome," published by Spencer Lens Company, Buffalo, N. Y.

Look at the cutting edge of the blade under the low-power objective of the microscope to examine for nicks. If the edge is appreciably nicked or not straight and even, it may be straightened by rubbing gently on a smooth stone, holding the knife at right angles to the stone. This will establish a straight flat surface along the cutting edge. Now hold the knife so that a strong nearby light reflects from the cutting edge. There will be a broad line of reflected light. Honing will remove small amounts of the metal first from one side of the edge and then from the other until nothing is left but the sharp cutting edge.

Use a fine hone or a piece of plate glass with appropriate abrasive. Lubricate the hone with a solution of neutral soap. Pass the knife over the stone, the cutting edge forward. In other words, proceed forward with the cutting edge of the blade, turn the knife over *across its back,* not over its edge, and bring it back, maintaining sufficient pressure to hold the edge evenly against the hone. If no pressure is used, the hone does not take hold well. If too much pressure is used, the edge is rounded. Gauge the amount of pressure in accordance with the hardness of the knife and the fineness of the hone. By watching the reflection of light from the edge of the knife, the progress of honing may be ascertained. When the reflection is very slight, examine the knife under the microscope to make sure that the edge is smooth and well sharpened.

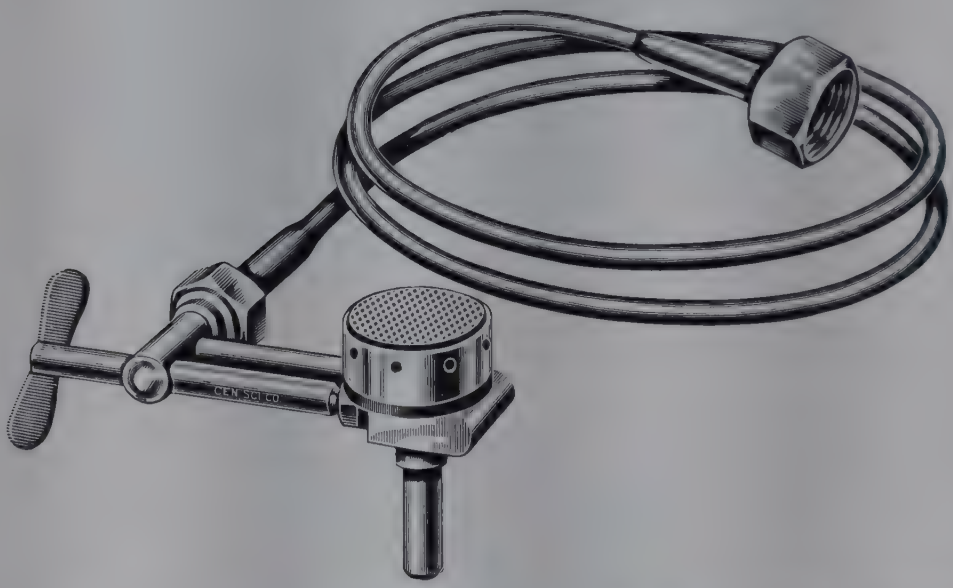

Fig. 610.—Spencer freezing microtome table. (Courtesy Central Scientific Co.)

The glass plate should be at least 3/6 inch thick or preferably thicker. Use plate glass 12 to 14 inches long and at least an inch wider than the length of the knife. The abrasive may be purchased graded to size; or it may be prepared by making a suspension in water and settling it in a tall cylinder. The heavy particles fall rapidly to the bottom; the lighter particles fall more slowly. The time of standing must be gauged by experience. Use the middle portion containing particles of proper size. For grinding, use an average size of 20μ, with none larger than 40μ. For final polishing of the facet, particles averaging 4μ and less than 8μ may be used. Suspend the particles in a solution of neutral soap (about 1 per cent). Gradation of abrasives is accomplished by pouring the supernatant liquid of the abrasive preparation serially from one container to another. The final supernatant liquid contains the finest particles.

To smooth the edge when nicks must be removed, No. 303 or 304 Corundum may be used. Levigated alumina or No. 305 Corundum is suitable for ordinary sharpening and after the above grinding compounds. Diamantine or similar material should be used for the final polishing of the cutting facets.*

To polish.—The knife is prepared as for honing. Use the finer abrasive. Very little is required. Proportions are soon learned by experience. Avoid too much abrasive. Wash the plate free from abrasive occasionally to rid it of large particles and any bits of steel freed from the edge of the blade. Hold the edge evenly against the glass; do not force

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*Dr. A. Klein, Norton Co., has suggested that a good polishing compound similar to Diamantine may be made by calcining pure ammonia alum in an *unglazed* clay or fused silica crucible for one-half hour at 2300° F. The crucible should be only one-quarter full as the alum swells during heating. The yield is about one-eighth of the original weight.

against it, or the edge will become round rather than sharp. If the facets are irregular, the grinding or polishing is uneven. Use fresh abrasives from time to time during the polishing operation. Toward the end use more dilute abrasives than at the beginning. Examine under the microscope. If the edge shows that the knife is well honed, stropping is unnecessary.

Stropping.—Do not strop too much, or the edge may become curved. Strop the knife from heel to toe. Clean the strop and free it from any abrasive larger than about 2 microns. The strop should be mounted on a board or held against some other surface so that it will not give as the knife is pulled over it. If the strop gives, or if the knife is pressed against the strop, the edge will be rounded and spoiled. Hold the knife firmly against the strop, with light pressure. After a few strokes, first on one side, then on the other, examine the edge under the microscope. Discontinue stropping just as soon as an edge appears smooth. It is unnecessary to test the knife with the fingernail, hairs, or other material; this only tends to dull the knife. The test of the knife is whether or not it cuts effectively.

If a knife needs touching up, but does not need a thorough honing, use a strop with one side embedded with diamond dust or other coarse abrasives. Then strop on the smooth side.

After the knife has been used, it must be cleaned and wiped dry, then put away in its proper place. If the atmosphere is corrosive, it may be necessary to oil the knife to prevent corrosion. Use only a good grade of light neutral oil.

If the knife is in such poor condition that it must be ground, it must be sent back to the manufacturing house which supplied it*; grinding should be done only by experts.

Krajian's Method of Sharpening a Microtome Blade.—

If the knife is dull, but free from nicks, it may be sharpened by stropping. Frequent stropping of the blade usually keeps it in good condition. If, however, the knife is used for a great many blocks, stropping alone will not keep it sharp enough, and one must resort to honing. If the knife must be ground, it should be sent back to the supply house where it was purchased;* grinding should be done only by experts.

For honing, use a number 108 carborundum stone. Before honing, cover the surface of the stone with fine machine oil. Be sure the back is in place on the knife, and that the handle is firmly screwed into the blade. Place the knife at one end of the hone, then, without any pressure except that caused by the actual weight of the knife, gently push it to the other end, so that the sharp edge is proceeding forward. Turn the knife over (against the back, not across the cutting edge) and push it back on its other side to the other end of the hone. Continue until the nicks are gone, or until it feels sharp to the thumb. This honing can be accomplished in about 5 to 10 minutes if properly carried out. If there are deep nicks in the knife, then exert firm pressure while honing.

Use a strop with both rough and smooth sides. When stropping, turn the knife over at the end of every stroke so that both sides are stropped in turn. Usually it requires about one dozen strokes on the rough side and about two dozen strokes on the smooth surface to obtain the proper cutting edge. Strokes should be gentle. Do not exert pressure. The weight of the blade is sufficient when stropping.

General Facts About Care of the Microtome

The microtome should be kept free from dust. Always cover it when not in use.

Be sure that all sliding parts are clean. Oil these parts occasionally with a good grade light machine oil. Be careful not to over-oil or to add the lubrication to dirty mechanisms. Place oil on the main bearing block through the oil inlet provided. Occasionally oil the feed screw.

Like all other precision instruments, the microtome requires gentle handling. Do not put undue pressure on the fly-wheel or other portions of the apparatus which are handled frequently. If the alignment of the microtome is interfered with by constant pressure at certain points, the instrument will have to be sent back to the manufacturer for repairs. To tighten connections, use a smooth wrench, not a pair of pliers. We have seen instruments literally "chewed up" with the erroneous use of pliers on the soft metal.

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*See footnote[1] on p. 2099.

After considerable use,[1] the microtome may cut sections irregularly due to wear. Turn the handle of the feed mechanism. A distinct drag or tension should be felt. When it turns freely it should be tightened. This usually stops the irregularity of the cutting. Tighten the friction screws on the friction bracket one full turn at a time until the friction is regained. If the feed pawl drags over the teeth of the ratchet wheel, adjust the friction block in the pawl lever by turning the two screws to be found back of the ratchet wheel. Turn these screws one at a time until the pawl lifts out, clear of the teeth of the ratchet wheel on the down stroke.

Do not disturb the feed screw mechanism. Exercise great care in returning the feed screw to its initial position after it has reached the end of its excursion and stopped advancing the specimen. Avoid use of force when handling the feed screw.

THE FROZEN SECTION METHOD OF TISSUE DIAGNOSIS[2]

It is essential to eliminate as completely as possible all lost time and lost motion from laboratory methods. The specialty is already so overcrowded with procedures that any saving of time, especially with promise of increased exactitude, should be welcomed by the practicing clinical pathologist and laboratory technician. The handling and examination of pathologic tissues have constituted for many years most important steps in the development of the hospital laboratory. Every piece of tissue removed at the operating table must be submitted to a pathologic investigation, both macroscopic and microscopic. In various places, one of three methods is used: the paraffin, the celloidin, or the frozen section. The celloidin method is used, so far as we know, in only a few places. The most common method in use is the paraffin method. The frozen section is used in some places as routine and in other places only as an emergency method.

The advantages of the frozen section method are as follows: (1) ease of manipulation; (2) saving of reagents; (3) lack of change in the normal structure of tissues, whereas in the process of paraffin embedding, it is impossible to prevent some shrinkage; (4) definite saving of time, which allows for more rapid diagnosis and permits the technician more time for other procedures; and (5) less expense in preparation. Slides are permanent.

Routine Frozen Section Method for Preparation of Permanent Slides (Krajian)

Technic of Freezing, Cutting, and Affixing Section to Slide.—

Fixation.—Ten per cent Formalin is used. Dilute 10 c.c. of commercial Formalin (40% formaldehyde) to 100 c.c. with tap water or saline. Neutrality of formaldehyde is important to preserve the staining qualities of the nuclei of the tissue. It is often necessary to add 5 grams of calcium carbonate or sodium bicarbonate to a gallon of the stock commercial formalin.

Immediate fixation is essential. Place the tissues in formaldehyde as soon as possible after removal from the body. At least 25 times as much fluid as bulk of tissues must be utilized. Pieces of tissue should not be too thick. Use a 15 per cent neutral commercial formaldehyde for 24 hours. **For rapid fixation** in cases where one is making a biopsy at the operating table, drop the tissue in boiling 10% Formalin for 30 seconds, then in dioxane for 30 seconds, and freeze immediately. For rapid fixation of autopsy material, use formaldehyde that has been brought to the bubbling point in a test tube, then pour it on the tissue in a bottle, which reduces the temperature of the formaldehyde to about 65° C. Allow it to remain in an oven at 56° C. for from 20 to 30 minutes. Shrinkage will be not more than 5%.

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[1] O. W. Richards: Loc. cit.

[2] Revised from the Pamphlet The Frozen Section Method of Tissue Diagnosis, compiled by R. B. H. Gradwohl, St. Louis, Mo., and A. A. Krajian, Los Angeles, Calif.

After 24 hours' *fixation* (with the slower method), the block should be cut in pieces about 1 to 2 cm. square, and from 3 to 5 mm. thick, and placed in 10% neutral commercial Formalin.

Washing.—Have handy a beaker or glass dish about 6 inches in diameter and about 3 inches high. Fill with tap water. Place the tissue blocks in the water for a short period only. This removes the formaldehyde which is detrimental to the skin of the operator and which would prevent the block from adhering to the holder.

The Microtome.—The Bausch and Lomb Optical Company freezing microtome is preferred. The knife should be honed and stropped in such a way that the edge has a shorter bevel on one side than on the other and should be used with the shorter bevel downward. The block holder should have deep grooves. Place the block on the holder after a drop of water has been placed on the holder. An angle of the block rather than a straight edge should face the knife. If the tissue is covered by a capsule, such as skin or a mucous membrane, this capsule surface must face the knife edge at an angle, and should never be placed on the side away from the knife.

To Freeze.—Place the index finger on the block and turn on the CO_2 gas slightly for temporary holding. Press down firmly but not tightly all around for fixing to the holder, and release more gas gently until the freezing level has passed upward through about one-half of the entire block. Excess water may be removed with a towel while freezing. Take care that there are no holes in the section, since the unfrozen portion will break; hence the importance of pressing the block to the holder firmly and releasing the gas slowly.

Cutting the Section.—Cut off the upper one-third, that is, the unfrozen portion, and drop it back into the specimen bottle. Then shave down to establish a smooth, level surface. If the surface to be cut is frozen too firmly, it is hard and white and the sections will be fragmentary. If the surface is too soft, the section will show holes. The ideal to be reached is a hard, pliable surface so that a section cut therefrom will show no tendency to break as it piles up over the cutting edge of the knife. If holes appear in the block while cutting, touch all parts of the cut surface gently with the fingers to warm it and melt it slightly, then refreeze. In this way, the holes in the unfrozen parts will become filled, and there will be no fragmentary parts of the tissue.

To Operate the Microtome.—Do not use a microtome where constant rotation of the wheel automatically lifts the holder. This faulty method, largely due to the manufacturer's wrong idea of making things look easy, is responsible for poor frozen sections. The author suggests that the automatic lift of the microtome be disconnected for frozen section work. *The hand must be used on the rim of the fly wheel* and the wheel must be turned slowly. Rapid use of the wheel, as is ordinarily carried out in laboratories, does not allow sufficient time to adjust the tissue surface to the proper temperature and consistency. The method recommended by Gradwohl and Krajian, i.e., slow turning by hand, permits warming of the cut surface of the block, giving a pliable section free from undulations.

Turn the notched wheel below the microtome table three (or two if desired) notches with the left hand, using the right hand to turn the fly-wheel. This will give a section about 8 to 10 microns thick. Uneven sections are usually the result of freezing the block too hard, applying too much speed and unnecessary force in cutting the sections, especially with firmer tissues.

Remove the Section From the Knife as follows: Wet the tip of the little or ring finger with water, and with this carefully remove the section from the edge of the knife (with a downward movement at about a 45 degree angle to the knife blade), and place in water. The tissue will float out on top of the water. Keep the knife edge dry. Water left on the edge thaws the tissue with which it comes in contact, causing a hole in succeeding sections. Place the sections in a large dish of water, about 3 inches deep, on a black background.

To Transfer Sections to the Glass Slide.—Clean the slide by dipping in acid alcohol and wipe dry. The dish in which the sections are floated should be at least 7 cm. deep and 15 cm. in diameter. The operator should be seated so as to prevent any direct reflection from the slide to the eyes. A glass rod, pointed or slightly bent, should be used to transfer the sections of delicate internal tissues to the slide. Teasing needles may be used for sections of firm tissues, but not when one is staining for iron or using silver solutions for staining. See Fig. 611.

Anchor the section to the slide at about a 45° angle, with three-fourths of it under the water in such a way that the edge of the water basin will afford support to either the slide or the fingers, thereby aiding in steadying the slide. Put the section, by means of a glass rod, on the middle of the slide, bringing it to the surface of the water immediately above the center of the slide. Now, manipulate the slide so as to straighten out most of the folds. Keep one edge of the tissue above the water. After the folds have been removed, tilt the slide against a staining dish, face down to keep off dust particles, at a 45° angle, so that the water may partially drain. *Do not permit it to become completely dry.* Fifteen seconds to a minute is usually sufficient.

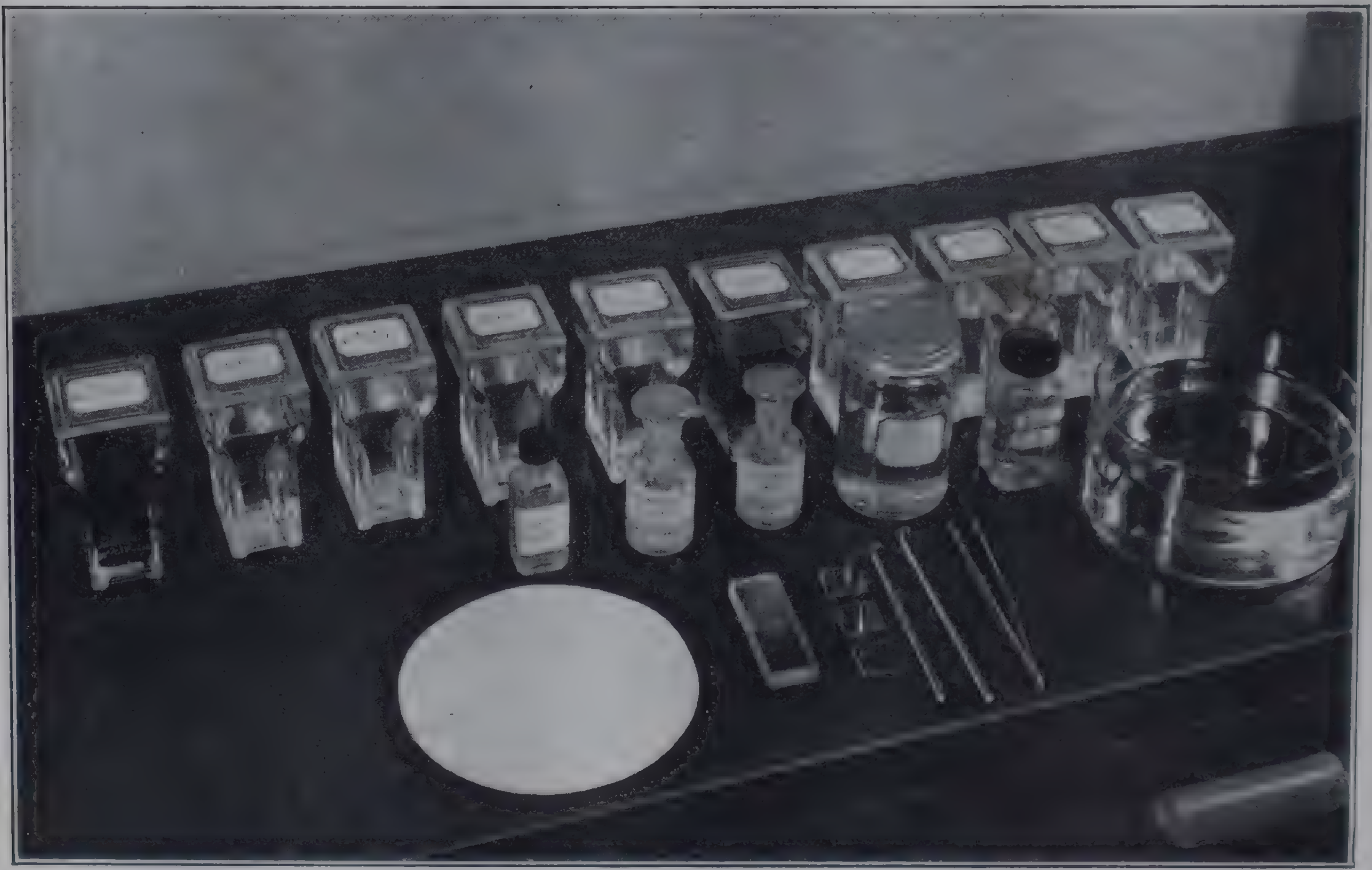

Fig. 611.—Equipment and reagents for staining frozen sections, method of A. A. Krajian.

To Dehydrate the Tissue.—Drop, at short range to prevent breaking the section, two drops of *anhydrous* isopropanol* or absolute alcohol on the section, holding the slide at a slight angle. Blow the breath over the section until the isopropanol or alcohol appears to have evaporated. Flood the slide with isopropanol or absolute alcohol for dehydration, for one minute or more. Pour off the dehydrating agent, taking care not to disturb the section. (In fatty tissues, fat is dissolved by the alcohol and the section may tend to slip away from the slide. Under these circumstances, blow over the surface of the section gently and remove the remaining alcohol by evaporation to keep the section on the slide.) Blot twice with several thicknesses of Whatman No. 1 filter paper, then dip twice (in and out rapidly but carefully, taking care not to change the position of the slide) into a jar of thin celloidin. (See below for technic of making.) Blow gently over the surface, taking care not to change the position of the slide *until the celloidin is dry.* When thoroughly dry, place immediately into the staining solution. Mayer albumin fixative is not recommened. Celloidin is that furnished by DuPont or Schering, or Parlodion, made by Mallinckrodt Chemical Works, St. Louis, Mo.

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*Isopropanol may be obtained from the Carbide and Carbon Chemicals Corp., St. Louis, Mo.

Thin Celloidin.—Make a *thick celloidin* (stock) by dissolving 30 grams of dry celloidin in 200 c.c. of a mixture of equal parts of ether and absolute alcohol. Shake the container several times a day for a week or longer, and stir the sediment with a heavy glass rod at least once a day until solution is complete. Then add about 7 to 8 c.c. of this thick celloidin to a mixture of 45 c.c. of ether, 45 c.c. of absolute alcohol, and 10 c.c. of acetone. When thoroughly dissolved, add 1 c.c. of saturated alcoholic solution of gum mastic to strengthen the adhesive qualities, make the sections smooth, and to prevent rapid deterioration (Krajian).

Staining Frozen Sections, Method of Krajian.—

Reagents for Staining.—

Harris Hematoxylin.—This hematoxylin formula is recommended as a nuclear stain. It is easily and quickly made.

Hematoxylin crystals	1 gm.
95% alcohol	10 c.c.
Ammonium or potassium alum	20 gm.
Distilled water	200 c.c.

Dissolve the hematoxylin in the alcohol. Dissolve the alum in the water by use of heat, and add the hematoxylin solution. Bring to a boil as quickly as possible, then add 0.5 gm. of mercuric oxide. The solution at once assumes a dark purple color. When this occurs, remove the vessel containing the solution from the flame and cool as rapidly as possible in a bath of running, or frquently changed, cold water. When cool, the solution is ready for use. Addition of 3 c.c. of glacial acetic acid to 95 c.c. of solution at the time of use will increase its nuclear staining properties.

Acid Alcohol.—Add 1 c.c. of concentrated hydrochloric acid, c.p., to 100 c.c. of 70% ethyl alcohol.

Ammonia-Isopropanol.—Add 5 c.c. ammonium hydroxide, c.p., to 100 c.c. of anhydrous isopropanol.

Eosinol.—See page 2133.

Carbol-Xylol.—To one part of melted carbolic acid crystals, add 3 parts of xylol. Be sure all glassware used in making the solution is dry.

Staining Technic.—

Solutions are placed in staining jars in proper order. Have on hand a drop bottle containing anhydrous isopropanol.

Put slide (section mounted under celloidin) in Harris hematoxylin for 3 to 10 minutes, or for rapid work, dip in and out carefully about fifty times or until the section is the proper color.

Place in tap water until blue (4 minutes).

Decolorize in acid alcohol by dipping the slide in and out of the jar, turning for even decolorization, until no more salmon pink solution runs off the tissue. Be sure to decolorize thoroughly, but do not over-decolorize.

Dip in tap water to stop decolorizing.

Place slide in ammonia-isopropanol until every part of the tissue is a good blue color. If desired, examine under the low power of the microscope to see if the nuclei are a good blue. Do not rush this step.

Pour anhydrous isopropanol over the slide (allowing it to flow into the ammonia-isopropanol jar to save it) to dehydrate the tissue. Several drops are sufficient, if repeated twice.

Dip in and out of eosinol several times, turning the slide for even staining, and watching the staining in the air over a white background until the section is stained evenly red. This requires 10 to 30 seconds or more, depending on the strength of the solution.

Place in carbol-xylol for 3 to 4 minutes, or dip in and out of carbol-xylol until clear (about 30 seconds).

Drain and place for 1 or 2 minutes in the first xylol, or dip in and out of the xylol for several seconds.

Place in the second xylol for 2 minutes, then into the third xylol to clear.

Mount in neutral gum damar. Do not allow xylol to dry before mounting.

Gum Damar.—

Gum damar is recommended instead of Canada balsam as a mounting medium because it presents several points of superiority: (1) It dries more quickly; (2) slides stay dry and can be stacked without sticking together; (3) it does not become yellow with age; and (4) it does not produce fading of the section.

Gum damar is a saturated solution of the gum in neutral xylol. Place in an incubator and shake it several times a day until it is of heavy syrupy consistency. Then filter through four layers of gauze and store in a brown bottle. If time permits, allow it to sediment thoroughly and decant the supernatant liquid instead of filtering. If the solution is too thin for mounting purposes, place the unstoppered bottle in an incubator until it is of proper consistency. If too thick, add more xylol.

Special Facts About This Method

In all cases where sections are transferred from one solution to another, be sure to drain off as much of the solution as possible, especially with carbol-xylol. Carrying of carbolic acid into xylols will cause fading of nuclear stains.

If the sections occasionally fall off from swelling, flood them with isopropanol for 2 to 5 minutes, pour off, blow section dry, blot, and dip twice in celloidin, and resume the process from that point on.

If sections do not clear properly, either the isopropanol is not anhydrous, or the carbol-xylol has been used too long, or the tissue has not been properly dehydrated. Try new solutions.

If the sections are cut unevenly, the microtome may be at fault, or the section was frozen too hard.

If a section has been re-celloidined, it must be dehydrated with isopropanol before placing in xylol.

When gum mastic is old or in solution, it deteriorates. It is necessary in such cases to use more gum mastic than the method calls for.

If the celloidin covering the section takes the stain during the staining process, the stain may be removed by dipping the slide back into thin celloidin until clear again.

Always be sure the celloidin has been blown dry before staining to prevent artefacts. The celloidin must completely penetrate the tissue before stain is applied otherwise the cells will not take the stain.

Air bubbles in the section form either because the section is too thick, there is not sufficient gum damar, or the gum is too thin, or the cover slip may not be properly laid down.

The celloidin manufactured by Mallinckrodt is fortified by the addition of gum mastic.

Hard tissues such as uterus and others of fibrous structure may be softened by the application of several drops of dioxane at the time of cutting. Then re-freeze. If the block is still too hard, the dioxane may be applied twice again. Never apply dioxane more than three times.

Soft tissues such as gelatinous carcinoma of lung tissue, if soaked in dioxane 15 to 30 minutes before cutting, give good consistency.

Striated muscle fibers are best shown with the spirochete stain.

If sections show shrinkage, it is usually caused by drying of the sections before they are dehydrated and celloidined. Watch every section very carefully during the draining process, and if any one is even slightly dried, dip that portion back in water and begin the dehydration immediately.

If the outside walls of appendices turn over, it may be due to the drying of the specimen before it reached the technician, and nothing can be done about it.

If the hematoxylin-eosinol stain is satisfactory, the nuclei will be clear under high power, the blues will be *blue* and not *purple* or *lavender*, while the eosin will be *pink* or *red*, but not *lavender* or *purple*. If the blues have a purple cast, the section was not properly decolorized in acid alcohol, nor the blue brought out properly in ammonia-isopropanol.

It is necessary occasionally to add more ammonium hydroxide to the ammonia-isopropanol to replace that lost by volatilization.

If the hematoxylin develops a sediment, pour off the supernatant liquid and continue to use it. A scum may be dissolved with a little glacial acetic acid.

If the celloidin peels off the slide or falls away from the tissue, this may be caused by using celloidin which is too old, or which is contaminated with extraneous material. Make new celloidin. Or it may be due to the fact that some of the solvent has evaporated, resulting in a thickening of the celloidin. Either thin the celloidin with a mixture of alcohol, acetone, and ether in proper proportion, or, better, make fresh thin celloidin.

Do not allow the section to dry at any step. If the section becomes dry, dip it back into the solution from which it has just been taken.

Frozen Sections of Loose Texture Tissues—Serumizing Technic (Krajian)

One of the principal objections to the use of frozen section methods has been the impossibility of securing satisfactory sections of fragmentary and loose-textured tissues, such as curettings, brain abscesses, and body fluids. Krajian[1] has overcome this objection by applying his serumizing method, as follows:

Fix in 10% Formalin (page 2102) in the usual manner for 24 hours, or longer if necessary. Trim the blocks. Wash several times in tap water to remove the formaldehyde. Place the tissue in a small Stender dish or specimen bottle containing uncontaminated human or animal serum. Fresh serum obtained from the day's serologic specimens is ideal for this purpose. The serum should completely cover the tissue. Cover the dish or bottle tightly and put in a warm place overnight. The top of the paraffin oven is a good place for it. Cover any floating tissues such as lung or fatty material with absorbent cotton in order to keep all parts submerged in the serum.

The following morning, decant the excess serum. Without washing, add sufficient dioxane to cover the blocks. Allow it to stand for 3 to 5 hours, or until the serum is completely coagulated. Immediately freeze on the freezing microtome with carbon dioxide gas, and cut, stain, and mount sections in the usual manner.

Since dioxane will eventually shrink the tissues, they cannot be kept in this solution indefinitely. After cutting the sections, place the blocks in 10% formaldehyde for permanent storage.

For emergency examination use very thin blocks of formaldehyde-fixed tissues. Place in serum in a paraffin oven at 56° C. for 1 to 2 hours, then coagulate in dioxane for one hour. Then freeze, cut, stain, and mount.[2]

This method has been used in our laboratories frequently. It causes very little shrinkage and no curling or shriveling of sections, such as other embedding processes intended for frozen sections. This serumizing process makes hard tissues, such as bone and uterus, soft and pliable, giving better sections.

Store the serum in small bottles and preserve by the addition of 2 c.c. of commercial 40% formaldehyde to 50 c.c. of the fluid. This keeps well at room temperature indefinitely without contamination.

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[1] Krajian, A. A.: Histological Technic, The C. V. Mosby Co., 1940.

[2] Krajian, A. A.: Am. J. Clin. Path. **10**: 87-88, 1940.

Frozen Sections at the Operating Table—Permanent Mounts (Krajian)

In most textbooks and in many hospitals, we see advocated a few different methods of frozen section for rapid biopsy work. The following method has proved very efficient at the Los Angeles County General Hospital and in our laboratory at the Christian Hospital at St. Louis:

Boil in 10% Formalin (page 2102) for ½ minute.

Drop in dioxane ½ minute.

Freeze and cut sections as directed on page 2103.

Place on slides.

Add a few drops of isopropanol (anhydrous) from a drop bottle, and blow dry. Repeat and let evaporate.

Cover the slide with isopropanol for about 30 seconds, drain off excess and blot between several layers of Whatman No. 1 filter paper.

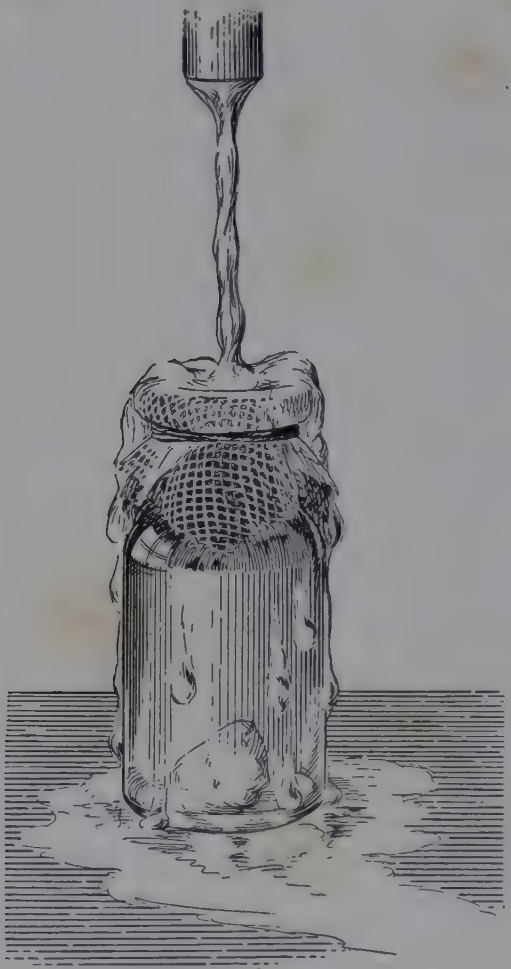

Fig. 612.—Washing tissues.

Dip into thin celloidin (page 2105) twice. Wipe the back of the slide dry. Blow the breath over the surface of the slide to dry the celloidin. *Do not change the direction in which the slide is held during the celloidining process until the celloidin is dry.*

Have all reagents in staining jars. See page 2105 for these reagents.

Dip in and out of Harris hematoxylin about fifty times.

Wash in tap water for a few seconds.

Decolorize in 1% acid alcohol as in the routine method.

Dip in tap water to stop decolorizing, and place immediately in the ammonia-isopropanol mixture until blue (5 c.c. ammonium hydroxide to 100 c.c. of anhydrous isopropanol).

Drop a few drops of isopropanol over the surface of the slide to dehydrate it, catching the isopropanol in the jar of ammonia-isopropanol mixture.

Dip in and out of eosinol several times until the tissue is a good pink color. This requires only a few seconds.

Dip in and out of carbol-xylol to clarify. About twenty or thirty dips usually suffice.

Dip in xylol for a few seconds, about thirty to fifty times.

Repeat in two more changes of xylol.

Remove excess celloidin from around the tissue, and mount in gum damar.

If the specimen becomes elevated on the slide at any time, dip in celloidin again, then back into xylol, and proceed.

AUTOTECHNICON METHOD FOR PARAFFIN SECTIONS*

The Autotechnicon is a special automatic apparatus especially designed for automatic fixation, dehydration, infiltration, decalcification, and staining of tissues. It can be adapted to any technic or method, and once it has been set, assures undeviating adherence to that technic.

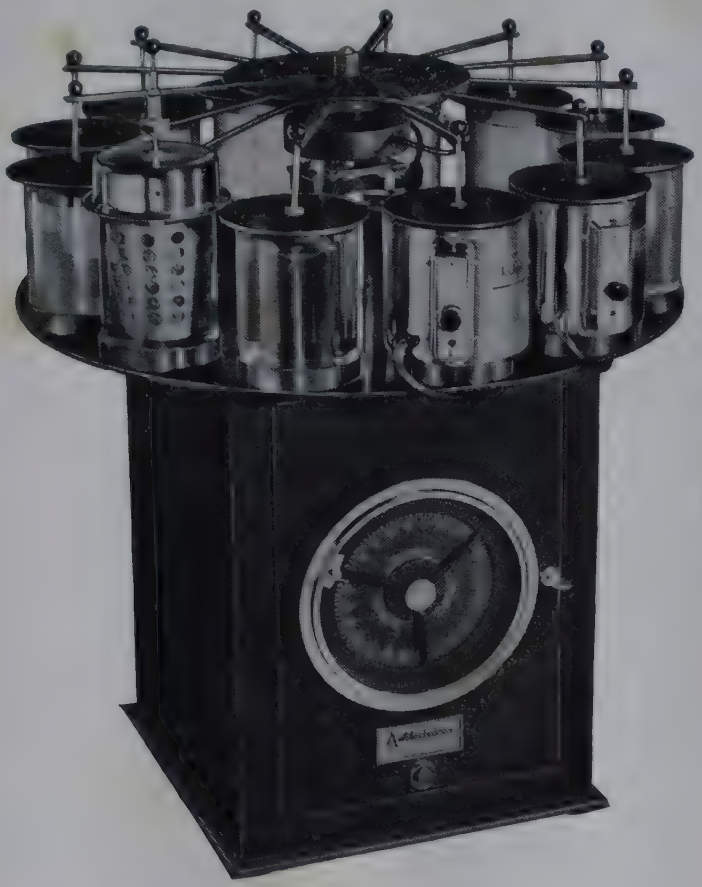

Fig. 613.—The Autotechnicon. (Courtesy Technicon Company, New York.)

The instrument consists of a timing clock, reagent beakers of polyethylene and Pyrex glass, beaker platform, mastershift carriage, beaker covers, displacer rotor, receptacle basket, receptacles, washer, and paraffin bath (Fig. 613). It is especially recommended for overnight tissue routine and for weekend tissue routine.

Suggested Daily Routine Technic, Using the Autotechnicon.—

Collect the tissues in the Technicon Tissue Caddy (Fig. 614) which can be wheeled to any location where it can be plugged into an electrical outlet, outside an operating room, in the morgue, etc.

Immediately upon excision, place the tissues in the refrigerated compartment. This prevents drying or general deterioration. The temperature within the Caddy is maintained at a point just below freezing so that tissues, while properly preserved, will still be easy to cut and trim on removal.

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*This section has been compiled from information furnished by The Technicon Company and Technicon Chemical Company, 215 E. 149th St., New York 51, N. Y.

When ready to process the tissues, roll the unit into the pathology laboratory, plug it into an electrical outlet, and store the tissues until they are ready for processing.

At a convenient time, the pathologist removes the tissues which have been collected during the day. As he prepares and examines each specimen, he describes its gross aspect, dictating his observations into a recording apparatus. It is convenient to have a permanent microphone set up at the cutting board, with a foot switch to start and stop the recorder, so that both hands are free for dissecting. While dictating the gross description, the pathologist cuts pieces of tissue to proper size for processing. These pieces should be cut no thicker than 4 mm., regardless of other dimensions.

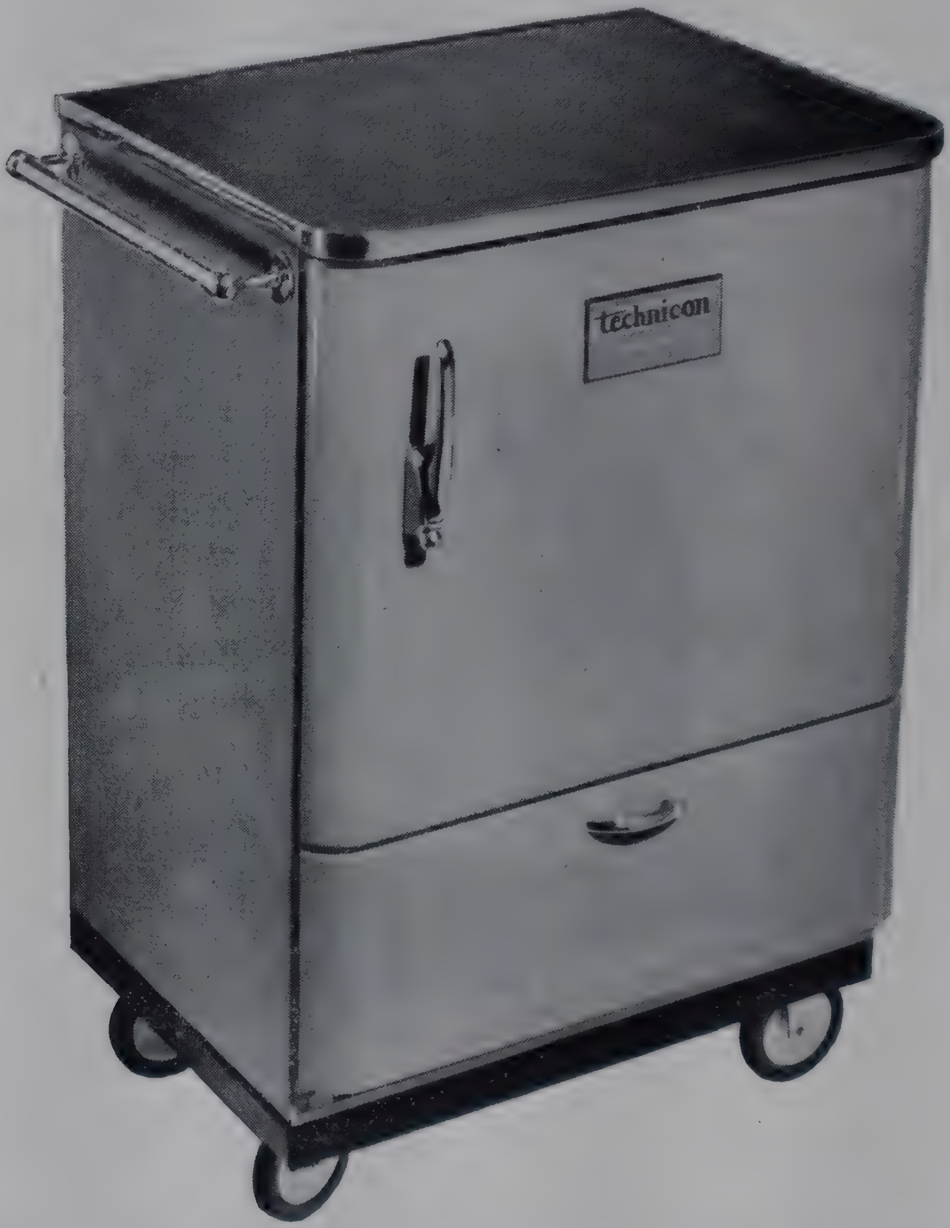

Fig. 614.—Tissue Caddy. (Courtesy Technicon Company, New York.)

As the pieces are cut, place them into a tissue receptacle fitted with proper dividers so that it can hold several specimens for simultaneous processing. During this procedure, store receptacle bottoms on one tray, dividers on another, and tops on a third. The pathologist can then select the tissue, pick the proper dividers, place them in a receptacle, and finally snap on the cover in a continuous operation.

If the pieces are small, the receptacle may hold three; if larger, two; while very large ones may require the entire receptacle. Place a paper identification slip, or slips, in the receptacle along with the tissue. Slips should be no larger than 3 by 10 mm. so as not to impede penetration of the fluid. Notations made with an ordinary graphite pencil will stand up under all the reagents used, and will still be quite legible when finally they come out of paraffin.

As each receptacle basket is filled, attach the displacer rotor to it, and mount the entire assembly, including the beaker of fixative in which it has been lying, on the Autotechnicon.

Check to see that no paraffin adheres to any of the beaker covers. This might solidify, and cause the beaker to tip and spill when the carriage rises.

Start the machine. Since precise time periods for each step have been cut into the timing dial, the process is now completely automatic. (See directions supplied with the apparatus.)

In a typical routine, the tissues will remain in the fixative for 4½ hours. The fixative which is recommended is Technicon fixative. It has all the advantages of Zenker fluid but none of its disadvantages, and is stable. Although it is a potassium dichromate formula, tissues fixed in it do not have to be washed since it produces no appreciable precipitate. It does not harden tissues. Tissues may remain in it for prolonged periods without harmful effects. In order to wash off the excess fixative and to avoid contamination of the reagents that follow, subsequent immersion in a beaker of water, or the tissue washer, for 30 minutes is recommended.

The next step is dehydration, with tissues remaining in each change for 1 hour each. Use the Technicon dehydrant, which is neither hygroscopic nor highly volatile. It does not cause tissue shrinkage, nor does it harden the tissue. Tissues may be left for prolonged immersion without damage.

Next, the tissues must be cleared through two stages, 1 hour each. The Technicon clearing agent is an excellent medium. It has the same qualities as the dehydrant, being neither volatile, hygroscopic, nor markedly inflammable, and having no shrinking, distorting, or hardening action on the tissues. It removes all the dehydrant, and conditions the tissues so that they will cut easily. It is highly soluble in paraffin. It renders the tissues translucent.

The tissues next go for 1 hour into the first paraffin bath, then for 2 hours in a second paraffin immersion. Two paraffin baths must always be used. The first becomes contaminated, but the second remains free from contaminants. The paraffin should have a melting point of 54° to 58° C.

If this process was begun at 5:00 P.M., the tissues will be ready for embedding at 9:00 A.M.

Lift the paraffin bath with its load of tissues in the receptacle basket and carry it to the embedding bench. Plug the bath into a convenient electric outlet to maintain the paraffin at the desired fluidity.

Remove the tissue receptacles one by one from the paraffin.

Lay a receptacle on the table, bottom down. With the forceps handle, strike the top lightly, causing the clamping lugs to spring open with a snap that breaks loose any adhering paraffin.

For embedding, use paraffin with a melting point of 60° to 62° C. Keep it fluid by heating over a Bunsen burner.

Use a flat pan for embedding. Coat lightly with glycerin before pouring in the paraffin so that the final paraffin slab can be easily removed after embedding. Pour in the melted paraffin.

Remove the tissues with a pair of forceps, transferring the tissue and its label to the pan of paraffin with the side to be cut facing down. Place the tissues in straight rows so that neatly squared blocks can be cut when the paraffin slab is divided for sectioning.

Set the pan on a fairly high stand and rub ice along the bottom so that the paraffin solidifies locally, anchoring the tissues firmly in place. The pan should contain about half an inch of paraffin.

To prevent crystalline formations, cool the slab slowly, by allowing it to stand at room temperature until solidified.

Place in cooled water for hardening. Do not use too low a temperature, as this causes the paraffin to form crystals.

Free the hardened slab from the pan and cut into individual blocks, each containing a piece of tissue with its label. Use the Technicon paraffin knife. The hot edge scores the slab deeply so that it is easily broken into individual blocks. (See Fig. 615.) Use the hot knife for final trimming of the blocks. Place the side of the knife against the side of the paraffin block, and as the paraffin begins to melt it can be easily made into a square block.

Mount the tissue block on the microtome object disk in the usual manner after removing the label with the heated paraffin knife. Keep the label with the tissue for identification.

Cut in the usual manner. The microtome blade is adjusted so that there is a 5 degree angle between the side of the paraffin block and the bevel of the microtome knife. A convenient method of determining this angle is to put the sharpening back on the knife, then hold a straight edge between the back and the edge of the knife to determine the exact angle.

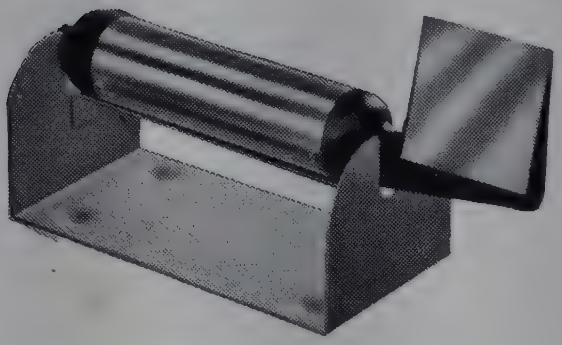

Fig. 615.

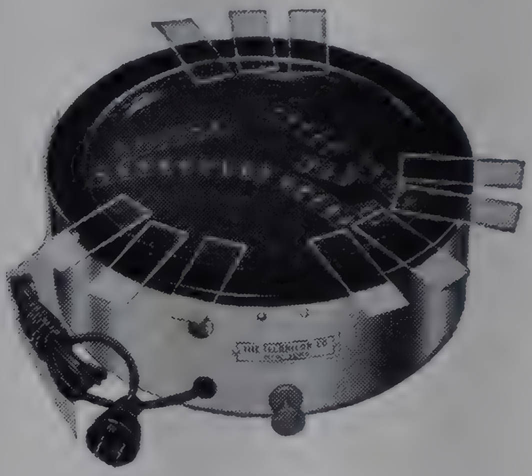

Fig. 616.

Fig. 615.—Technicon paraffin knife. (Courtesy Technicon Company, New York.)
Fig. 616.—Technicon water bath. (Courtesy Technicon Company, New York.)

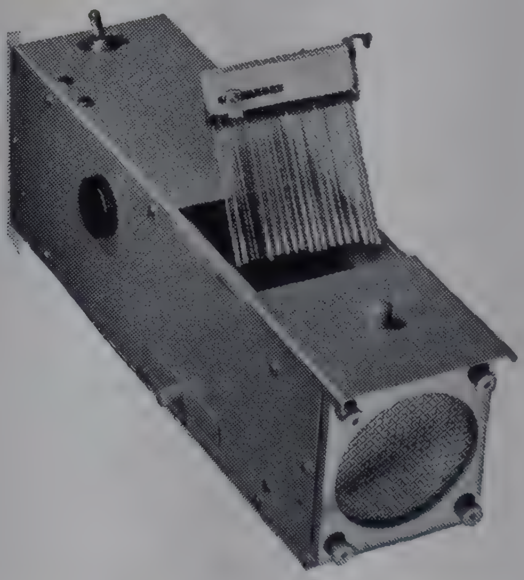

Fig. 617.—Technicon microslide dryer. (Courtesy Technicon Company, New York.)

Do not take preliminary thick trimming cuts. These rapidly dull the knife edge.

Always put the narrower side of the tissue block nearest the knife when inserting the block into the microtome.

As the cut tissues are lifted from the microtome, spread them face up on a Technicon constant temperature water bath (Fig. 616). Before placing a cut tissue section on a slide, identify the specimen by scratching the number into the slide, using a diamond point pencil.

Keep the water bath at 45° C. by adjusting the thermostat. Keep the water level near the top. Remove unwanted tissues by dropping a paper towel flat on the surface. When it is lifted, the paraffin ribbons will come away with it.

Mount the tissues in egg albumin fixative in the usual manner. If desired, dip the mounted slide in a container of very hot water, quickly, to flatten the tissue section. Use a temperature of 95° C., but be sure not to leave the slide in the water, or the paraffin will melt.

As the slides are mounted, insert in the staining rack, and place it in a Technicon microslide dryer. A current of warm (56° C.), filtered air passes over them, completely drying the slides in about 7 minutes (see Fig. 617).

To use the Autotechnicon for staining, plug in a one-hour staining clock, at the same time replacing the processing fluid beakers with others containing those staining fluids required by the prescribed technic.

Keeping the dried slides in the rack, take from the drier, and mount the rack on the Autotechnicon for staining. Operation from this point is completely automatic.

The tissues move first into a beaker containing xylol to remove the paraffin. This immersion is for 2 minutes. They then go into 0.25% iodine dissolved in the dehydrant, to remove any mercuric chloride crystals remaining in the tissue. This requires 2 minutes.

Next, a bath of Technicon dehydrant removes excess iodine, 2 minutes. Then distilled water 2 minutes, hematoxylin for 10 minutes (or longer if desired), a rinse of distilled water for 4 minutes, lithium carbonate to render the hematoxylin alkaline, turning the dye blue, 2 minutes. They then move into distilled water for 2 minutes, then eosin 5 minutes, distilled water to remove excess eosin, dehydrant for two baths of 5 minutes.

Remove the rack after dehydration, and place upside down in a beaker of xylol.

Remove the slides from the rack singly, clean, and mount under a cover glass.

Although any desired stains may be used, it is recommended that the Technicon standardized hematoxylin and eosin be used for the staining process. Technicon tablets of lithium carbonate are also convenient; dissolve 1 tablet in a liter of distilled water to obtain a 0.01 N solution.

The Technicon standardized hematoxylin and eosin will last approximately six months, after which their staining efficiency sinks to zero as the balance is destroyed. No subsequent decolorization is required when Technicon eosin is used.

Technicon mounting medium, Canada balsam, gum damar, etc., can be used as mounting media.

Other staining methods may be used by preparing another clock, according to directions furnished with the apparatus, and substituting other staining solutions.

PARAFFIN SECTIONING

Paraffin sectioning differs from the making of frozen sections in that, with this method, the water is replaced in the section by paraffin, then thin sections cut. In order to remove the water and entirely replace it with paraffin, the tissue, after proper fixation, must first be dehydrated. This is accomplished by carrying it through increasing strengths of alcohol, or isopropanol, beginning usually with 50 per cent strength. Thus the water in the tissue is gradually replaced with the dehydrating agent, until all water is removed, and 100 per cent alcohol or anhydrous isopropanol is present in its place. The alcohol is then replaced by xylol (which clarifies the tissue); the xylol permits the penetration of the tissue by paraffin, which then replaces the xylol. This entire process of dehydrating, clarifying, and embedding requires several days, at the end of which time the tissue is embedded in pure paraffin, then cut into sections of appropriate thickness, and stained.

Before the sections can be stained, they must first be affixed to the slide. Various methods of fixing sections are given below. Next, the paraffin must be removed from the section. This is accomplished by the use of xylol. Water must gradually replace the xylol now in the tissue. This is accomplished by passing the tissue through gradually decreasing strengths of alcohol or isopropanol until eventually the original water content of the tissue is restored. The section is now ready to be stained.

Staining processes are quite similar to each other. The tissue is first submitted to hematoxylin (an excellent nuclear stain), decolorized to remove hematoxylin from all portions of the tissue except the nuclei, the blue color brought back with an alkali, then the tissue counterstained with a contrasting color (red, of the eosin); the nuclei remain blue; other portions take the eosin dye.

The tissue must again be dehydrated, since it is to be clarified and mounted in a material which is immiscible with water. This is accomplished by employing increasing strengths of alcohol, or isopropanol, and finally xylol.

Details of the methods follow.

Formulas for Fixatives (or Tissue Hardeners)

It has been found by Stowell[1] that isotonic saline is superior to distilled water as a Formalin diluent, reducing tissue swelling during Formalin fixation. Reagents producing marked decreases in tissue volume render such tissues less susceptible to shrinkage during subsequent procedures.

Alcohol, 96% or 100%.—

When alcohol is used as a fixative, use very small tissues and keep in alcohol at least two days, changing the alcohol frequently.

Formalin.—

We ordinarily use a 5% or 10% solution (made from the 40% gaseous solution), 5 or 10 c.c. of 40% formaldehyde to each 100 c.c. of solution.

Formol-Alcohol.—

Alcohol, 80% to 100%	2 parts
Formalin	1 part

Susa Fixative.—(See page 2170.)

Orth Mixture.—

Formalin	1 part
Mueller solution	9 parts

(Mueller solution is made up as follows: Bichromate of potassium 2.5; sodium sulphate 1; water 100.)

Acetic Acid Fixative.—

(a) 1 part acetic acid
3 parts absolute alcohol
(b) 1 part acetic acid
6 parts absolute alcohol
3 parts chloroform.

Solution (b) is good for lymph and glandular tissues. The chloroform helps penetration. Tissue is fixed more rapidly. When using alcohol-containing fixatives, omit all steps preceding the solution of the strength of alcohol used.

Wash out the acid-alcohol with 95% alcohol.

If tissue is submitted in alcohol, put it in 95% alcohol or the acid-alcohol solution above, as the exact strength of the alcohol fixative must be known.

Bouin's Fixative.—

Saturated aqueous solution of picric acid	75 c.c.
40% formaldehyde	25 c.c.
Glacial acetic acid	5 c.c.

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[1] Stain Technology 16: No. 2, April, 1941.

This has good penetration powers. Picric acid present in the fluid, which stains the tissue, is extracted in the process of dehydration.

Zenker's Fluid.—

STOCK Zenker's fluid (fixative)

Potassium bichromate	2.5 gm.
Mercuric chloride ($HgCl_2$)	5 to 8 gm.
Distilled water	100 c.c.

Dissolve by heat.

Zenker's for use.

Glacial acetic acid	5 c.c.
Stock Zenker's	100 c.c.

Mix when ready to use.

Technic when using Zenker's fluid.—

Fix twelve to twenty-four hours, dependent upon the size of the tissue.

Wash in running water twelve to twenty-four hours.

35% alcohol	20 minutes.
50% alcohol	20 minutes.

70% alcohol with enough iodine to give it a good port-wine color. (The iodine removes the mercury.)

Keep in this solution until all the color of the iodine disappears.

Renew the iodine after it disappears and leave the tissue in the solution.

Continue the renewal of the iodine until the tissue no longer decolorizes it. At this point all mercury has been removed from the tissue. This step is very important, as the ensuing processes cannot be carried out successfully if any mercury remains in the tissue.

70% or 80% alcohol until all color (iodine) comes out of the tissue.

Proceed from this step as in the long tissue work.

Fleming Solution.—

Chromic acid 1%	15 parts
Osmic acid 2%	4 parts
Acetic acid	1 part

Use very thin pieces of tissue. Fix several days. Wash twenty-four hours in running tap water.

Regaud's Fixative.—(See page 1407.)

Solutions for Mounting

1. **Canada Balsam.** A good grade of Canada balsam for mounting slides may be purchased from any reliable chemical supply house.

2. **Gum Damar.** Dissolve gum damar in xylol until thin enough for use. (See page 2106.)

3. **Euparol** may be used. It is either colorless or green. Use the green for hematoxylin-eosin stains only. It intensifies the microscopic picture. Very often poorly stained sections mounted in euparol (green) are intensified after mounting. When mounting in euparol, take the tissues directly from the 95% alcohol, omitting the absolute alcohol and xylol steps.

Methods of Fixing Paraffin Sections to Slide

Mayer's Albumin Fixation:

This method was first described by Von Henniguy. It was later modified by P. Mayer, and still later by Von Reinke.

Beat the white of an egg until stiff and dry. Let stand until clear.

Filter through filter paper.

Add an equal volume of glycerine.

A crystal of thymol may be added as a preservative if desired.

To use this fixative, the solution is rubbed briskly over the surface of a slide, but not allowed to dry. The paraffin section is teased onto the slide in a drop of water. The excess water is removed by gentle blotting, and the section is placed in an incubator at 37° C. overnight, or gently warmed in the flame.

Preferred Method:

Use equal parts of egg white and glycerine. Beat thoroughly with a glass rod and filter through several thicknesses of gauze. Add crystal of thymol as a preservative.

Alternate Method:

Equal parts of blood serum and glycerine are mixed with 10% Formalin. To use, a faint trace of the mixture is placed on a slide.

Warm gently in a Bunsen burner, keeping the heat at about 70° C.

Pour distilled water over the slide.

Next the section is placed in the distilled water, and straightened.

The excess water is removed with filter paper.

Place the slide in the incubator at 37° C. for three or four hours,

or

Have the section in slightly warm distilled water.

Float a cover glass on the section to pick it up.

Heat the preparation to 70° C., and continue as described below.

Two Time-Saving Methods of Fixing Sections to Slides:

Two very important improvements in laboratory technic have been reported by Schneidau.[1] These methods possess distinct advantages over Mayer's albumin fixation method commonly used in laboratories for fastening microscopic sections to slides.

The first method described is as follows:

Albumin Solution.—

Egg white	one
Glycerin	50 c.c.
Sodium salicylate	1 gm.
Distilled water	1500 c.c.

Mix thoroughly and filter. Place the solution in a narrow-mouth jar equipped with a two-hole rubber stopper. Through one hole fit a bent-glass siphon to which has been attached a rubber hose containing a small pipette at the end, and a spring clamp. Place the apparatus on a shelf several feet higher than the laboratory table, start the siphon, then shut off the flow with the spring clamp. To use, assemble slides, squeeze the clamp, and allow just enough fluid to run onto each slide to float the section. Float sections by means of a section lifter, spread out by heating, and blot up excess fluid. Set aside to dry. With this method the danger of using too much or too little fixative is practically eliminated. The sections do not entirely dry, due to the glycerin present, but may be left on the slides for months before mounting without danger of drying or cracking.

A second method consists in floating the sections onto the surface of slightly warmed water to spread them out and then placing them on the slides and drying, without the use of albumin at all. Specifically, the procedure is carried out as follows: Use a pan of approximately 15″ × 10″ × 3″. Fill the pan with water and heat until it feels fairly warm to the hand (about 40° C.). While the water is being heated, cut the ribbon into squares of one section each. Take the sections up one at a time on a section lifter and drop them onto the surface of the water. Be sure that one section does not fall on top of another, as they cannot be separated once they are in the water. When all the sections that are to be affixed have been dropped on the water (no more than 100 should be worked at one time in a pan of the dimensions stated above), dip a slide into the water at an angle of about 50° until the water level is about ½ cm. above the midline of the slide. Take a straight probe or needle and, catching any of the sections by one edge, pull it over and hold it against the slide with the needle and pull both slide and needle out of the water together. The water will run out from under the section and leave it in about the center of the slide (depending, of course, upon how close to the center the section was held with the needle). This procedure is repeated until all of the sections have been placed on slides. The slides are then placed in a drying oven overnight to dry. If no drying oven or incubator is available, then the slides must be left alone for three days to insure complete evaporation of the water. Contrary to the belief of many, the sections will not wash off the slides provided they have completely dried and do not receive rough handling in the staining and mounting processes. One precaution to be observed is to see that there are no bubbles on the surface of the water that is used, as a bubble under a section will cause it to crack when dry or to wash off of the slide. This method is essentially a time-saving method, but is not as reliable as the first method described. However, if done carefully it should not cause any trouble.

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[1] Tr. Am. Microscopical Soc. 56: 258, 1937.

General Considerations of the Formalin-Alcohol-Paraffin Method

This method can be used on all tissues.

Formalin is used because it kills, fixes, and hardens the tissue. This must be done so that the tissue remains intact, undergoing no further change. Use 10% Formalin. Formalin fixes quickly, does not shrink the tissue, nor expand it. Few tissue changes occur. It penetrates all parts equally well, including fat. It properly prepares tissue for subsequent processes.

The second step is washing in water. Use running water, or put the tissue in a bottle, changing the water frequently. All Formalin must be removed. Osmosis is the underlying principle by which penetration is accomplished by the various solutions; therefore, the steps must not be taken too rapidly.

In the dehydrating process, low-grade alcohols or weak isopropanol solutions are used first. Water is removed because paraffin is to be substituted later. Alcohol (or isopropanol) is the best dehydrating medium.

Increasing strengths of alcohol (or isopropanol) are used until the tissue is in absolute alcohol (or anhydrous isopropanol).

When the tissue is in absolute alcohol, it is too opaque for examination. It is clarified in xylol. The xylol displaces the alcohol readily and aids in the penetration of paraffin into tissue. If there is any water left in the tissue, the xylol becomes turbid; replace tissue in the absolute alcohol and transfer back to xylol again if this occurs.

Xylol and paraffin mixture is used to begin the penetration of paraffin.

Paraffin is used to complete the penetration.

The eighth step is embedding in paraffin preliminary to cutting the section.

Cut, place on the slide, and stain.

Mount and label.

Embedding Methods

There are various embedding methods useful in histopathologic procedures. Of these, paraffin embedding is most widely used. It is particularly useful when very thin sections are desired. Tissue blocks with this material should be small, not more than 2 to 3 mm. thick. Tissues are placed in paraffin, after being dehydrated either with graded alcohols or isopropanol, and after being placed in a mixture of chloroform, xylol, or benzine. The advantages in the use of chloroform as compared with xylol are that it enables the paraffin in the mixture to partially infiltrate the tissue, and then it rapidly evaporates from the pure paraffin bath. Pure paraffin of a definite melting point is used in these procedures; the so-called ''Fisher Tissuemat''* has been used with great success. This is an embedding compound superior to the paraffin usually employed in that it will not crumble or crack; it adheres to the specimen and enables a thinner section to be cut. It is ideal for making ribbon sections. It is supplied at various melting points. Our own preference has been that with a melting point of between 56° and 58° C. Another excellent paraffin product is Histowax.† If either Tissuemat or Histowax is used for embedding, it is substituted throughout the entire procedure.

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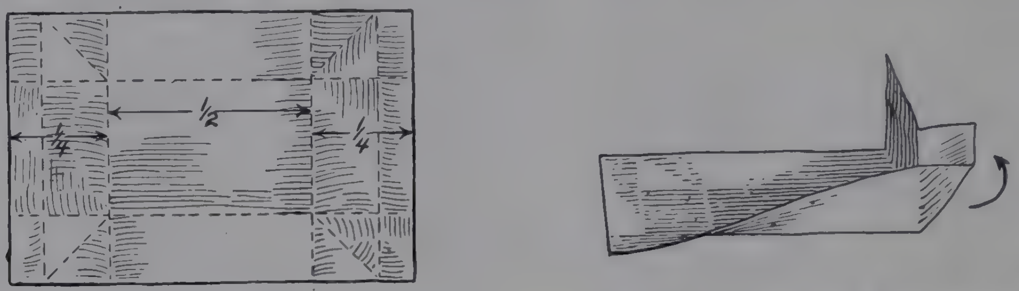
*Manufactured by Fisher Scientific Company, Pittsburgh, Pa.

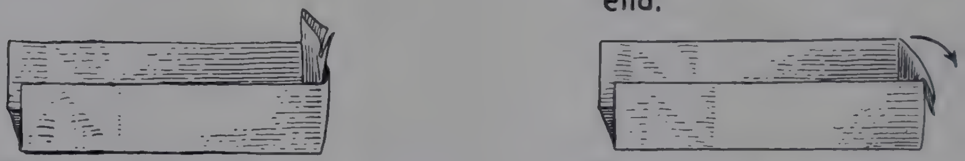
General Facts About Embedding Paraffin.—

Special paraffin is manufactured for embedding purposes. Different samples of paraffin have different melting points, and also different "plastic points."[1] Paraffin is a mixture of hydrocarbons which solidifies into characteristic types of crystals, varying to some degree with the proportion of harder and softer hydrocarbons present. The peripheral crystals are oriented with respect to the cooling surface while the center of the mass forms a meshwork (Dempster, 1941). The "plastic point" is the lowest temperature at which permanent deformation may be made without fracture. A paraffin with low plastic point appears more translucent, is less brittle, but compresses more in sectioning. The hardness of paraffin depends on its plastic point which lies a few, but variable number of, degrees below the melting point. Consequently, the plastic point or, more roughly, the melting point of the paraffin has to be adapted to the temperature of the room in which the sectioning is being done.[2] In warm rooms, higher melting point paraffins must be used than in colder laboratories. The alternative is to condition the paraffin by the addition of various materials. Bayberry wax, rubber, and other waxes may be added to paraffin to improve its plasticity and cutting qualities.



① Crease on dotted lines

② Fold up one end and one side. Fold projecting flap against the end.



③ Repeat with adjoining corner.

④ Fold over and crease the projecting end-flap to lock the corner joints.

Fig. 618.—Method of making "boats" for embedding paraffin sections.

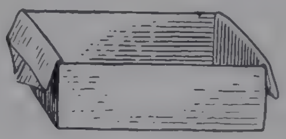


Fig. 619.—The completed "boat," ready for use.

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†Matheson-Coleman & Bell Division, The Matheson Company, Inc., E. Rutherford, N. J.

[1] We are indebted for the following material to O. W. Richards, The Effective Use and Proper Care of the Microtome, published by American Optical Co., Scientific Instruments Division, Buffalo, N. Y.

[2] Washbotton in Bensley and Bensley (1938). Bensley, R. R., and Bensley, S. H.: Handbook of Histological and Cytological Technique, 1938, The University of Chicago Press, ch. 8.

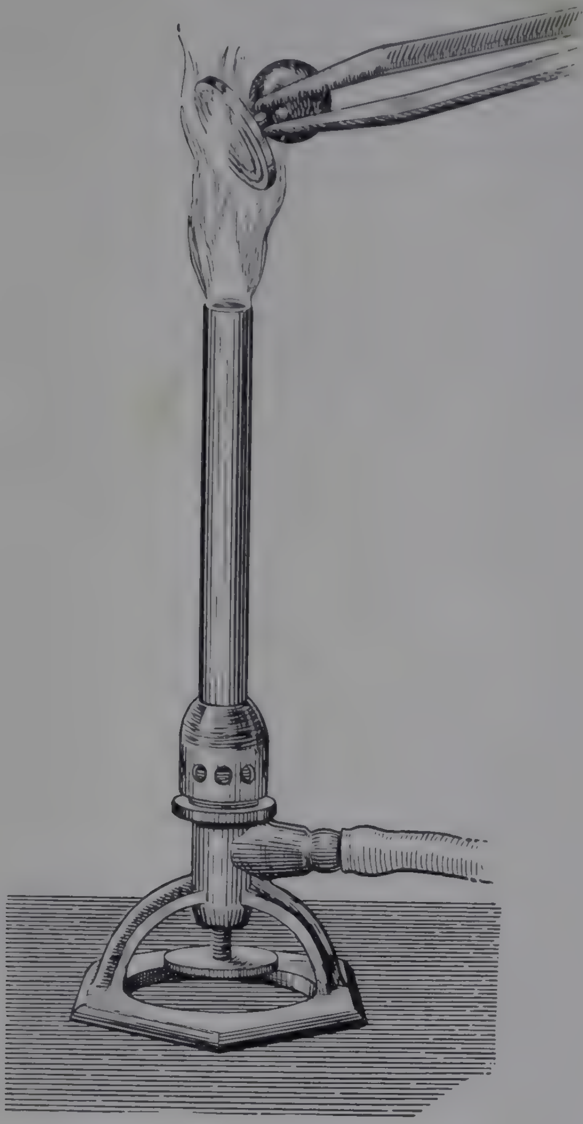

Fig. 620.—Heating the paraffin microtome pivot.

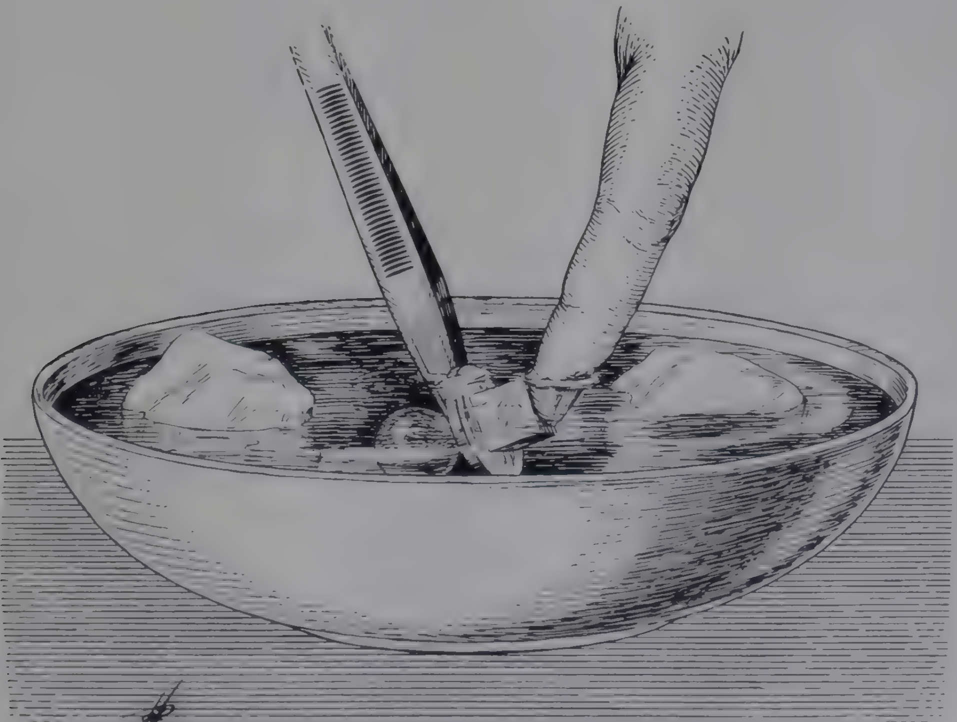

Fig. 621.—Cooling the paraffin block after mounting it on the pivot.

In addition to matching the hardness of the paraffin to the temperature at which it is to be sectioned, it is necessary to match the hardness of the paraffin to the hardness of the tissue. The hardness of the tissue depends upon the preparatory treatment and its structure. The actual process of sectioning leads to distortion so that the sections will be slightly thicker and a little shorter than the block itself. This distortion results from the nature of the paraffin, the tissue, and the action of the knife. Large crystals are sectioned or pushed apart by the wedging of the knife, especially when the paraffin is brittle and has a high plastic point. This upsetting of crystals gives the velvety appearance to the topside of the section.

When the crystals are not of proper size to fit closely to the tissue, they cannot support the tissue adequately, and local deformation will occur, giving a section which cannot be completely flattened out. Small folds show when the thin parts are smaller than the size of the paraffin crystals and compress less than the paraffin. The crystals show clearly in polarized light. All microtomists should examine a section from each batch of paraffin with a polarizing microscope. A finer crystalline paraffin gives adequate support.

Warm paraffin shrinks as it cools and compresses the tissue in the block. Tissue that is harder than the paraffin withstands this pressure, but soft or spongy tissue may be under considerable strain. When sectioned, the tissue tends to expand to the shape and size it had before compression, and if confined by the paraffin around it, pleating or wrinkling results. Stowell[3] reported that Parowax shrinks in volume 14.3 per cent on cooling from 59° to 20° C.

Unless the hardness of the paraffin is adapted to the temperature at which the cutting is done and the nature of the tissue, good sections may not be expected, regardless of the excellence of the microtome and sectioning knife. When the material has been properly dehydrated, it is possible to re-embed it in another paraffin should the first not prove satisfactory. Poorly prepared tissue can rarely be salvaged. Several changes of the melted paraffin should be used to wash out the former paraffin.

TECHNIC OF EMBEDDING AND CUTTING PARAFFIN SECTIONS

Alcohol Dehydration Method for Paraffin Embedding

Reagents.—

10% Formalin.—See page 2102.

50% Alcohol.—Dilute 50 c.c. of 95% alcohol to 95 c.c. with distilled water.

70% Alcohol.—Dilute 70 c.c. of 95% alcohol to 95 c.c. with distilled water.

80% Alcohol.—Dilute 80 c.c. of 95% alcohol to 95 c.c. with distilled water.

100% Alcohol.—Absolute alcohol can be kept at 100% concentration by mixing it with pulverized copper sulphate which has been previously dehydrated. The copper sulphate falls to the bottom of the bottle, the 100% alcohol remaining above.

Xylol.—Purchase chemically pure or U.S.P. xylol (xylene water- and acid-free).

Xylol-Paraffin Mixture.—Mix 1 ounce of pure xylol with 1 ounce of special paraffin, and keep in a warm (not hot) place.

Pure Paraffin.—We suggest the use of pure paraffin, melting point 56° to 58° C., made by Arthur H. Thomas Co. or Matheson Coleman & Bell Inc., or Fisher Scientific Company.

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[3] Stain Tech. **16**: 67, 1941.

Fisher Tissuemat.—This is an embedding compound manufactured by Fisher Scientific Company, Pittsburgh, Pa. It is superior to paraffin because it will neither crumble nor crack when being cut on the microtome. It is well adapted for ribbon sections since it coheres during slicing and facilitates the cutting of very thin slices. This material is also more convenient to use than paraffin cakes because it is supplied in granular form. It melts rapidly and obviates the necessity of cutting large cakes. The granules are protected by an inner container of cellophane.

"Tissuemat" is available in five melting point ranges.

Histowax.—Histowax is an embedding compound manufactured by Matheson-Coleman & Bell Division, The Matheson Company, Inc., E. Rutherford, N. J. It is an excellent substitute for paraffin, will not crumble or crack during the cutting process, and is well adapted for making ribbon sections.

Mayer's Albumin Fixative.—See page 2115.

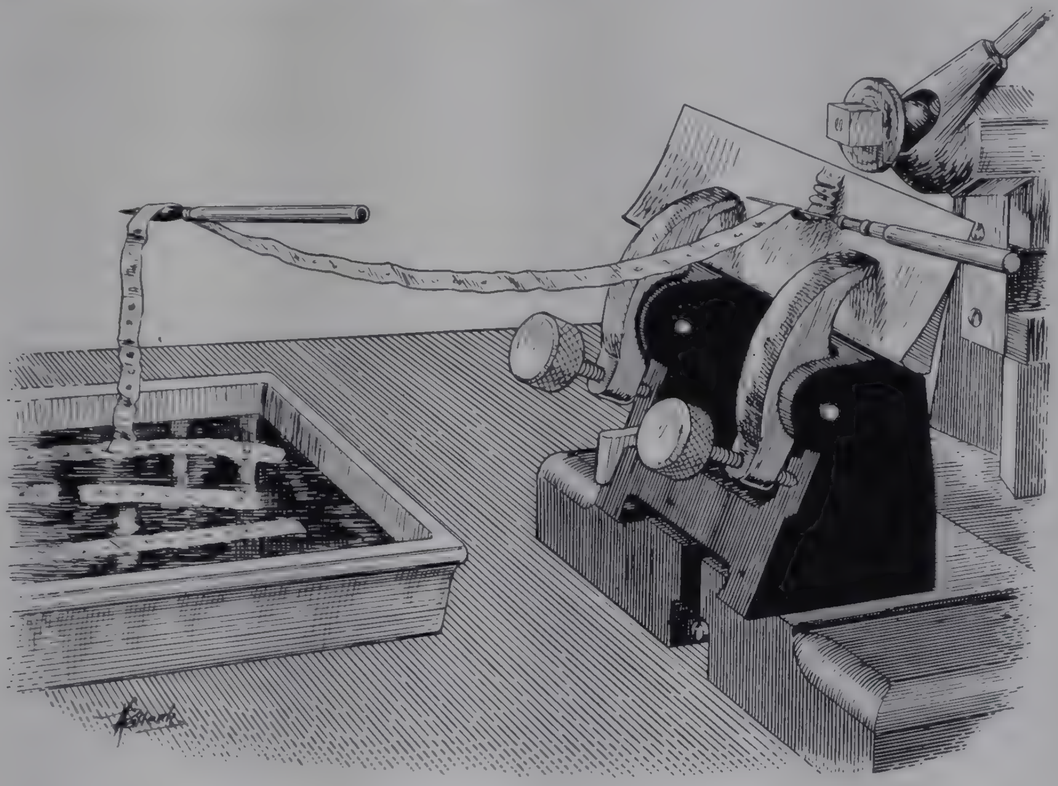

Fig. 622.—Cutting paraffin sections and floating the ribbons on warm water.

Technic.—

1. The pathologist removes the portions of tissue for examination. These should preferably be not thicker than ¼ inch. Keep in Formalin (10%) *at least* two hours. After a month in Formalin, it is difficult to treat the section.

2. Wash one hour in tap water, or a shorter time in running water to remove the Formalin. The tissue may be kept in the same bottle throughout the dehydrating and clarifying processes.

3. 50% alcohol[1]	1 hr. (longer, if necessary. Stopping point is one hour)
70% alcohol	1 hr. (longer, if necessary. Stopping point is one hour)
80% alcohol	1 hr. (longer, if necessary. Stopping point is one hour)
95% alcohol	1 hr. (longer, if necessary. Stopping point is one hour)
100% alcohol	not more than 1 hour.

These solutions may be used repeatedly until a sediment appears.

4. Xylol one hour to clear. If not clear, immerse for a longer period. It is opaque until placed in xylol, when the edges become clear. If there are milky or chalky spots in the tissue, put it back in 95% alcohol, then absolute alcohol, then xylol.

5. Xylol-paraffin (equal parts)—all night with just enough heat to keep liquid. DO NOT PUT IN THE PARAFFIN OVEN.

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[1] Isopropanol of appropriate strength may be substituted for alcohol in all dehydrating processes, but *not* in the staining solution formulas.

6. Next morning. Pure paraffin two to two and one-half hours at 58° to 60° C.

7. Pure paraffin again. Tissues can be left here a day if necessary. (Four to five hours is all that is necessary.) The oven must not be hotter than 58° to 60° C.

8. Embed or orient. Use paper box or "boat." This may be made of a piece of paper or piece of tin foil about 0.1 mm. thick. This is easily done by folding the tin foil around a small block of wood with rectangular surface or by following directions illustrated in Figs. 618 and 619. Fill this container almost full with paraffin. Transfer the object into it with the aid of a warm spatula. Arrange it in the center of the container and have the whole float in a bowl of cold water. The paraffin congeals rapidly. After the first skin of paraffin appears on the surface, the "boat" may be dipped into the water. In a few minutes' time the paraffin is rigid and the tin foil may be removed. The paraffin block with the enclosed object may be kept for any length of time.

Place the section in paraffin with the side to be cut facing the bottom of the box. Float in cold water until a firm scum appears on the top of the paraffin. Submerge in the water and hold there until the paraffin has hardened. Cut out blocks containing the tissue, leaving an edge of paraffin about 1 cm. wide around the tissue. Opposite sides should be cut parallel. The block should be square or rectangular. Edges parallel to the knife must be parallel to each other.

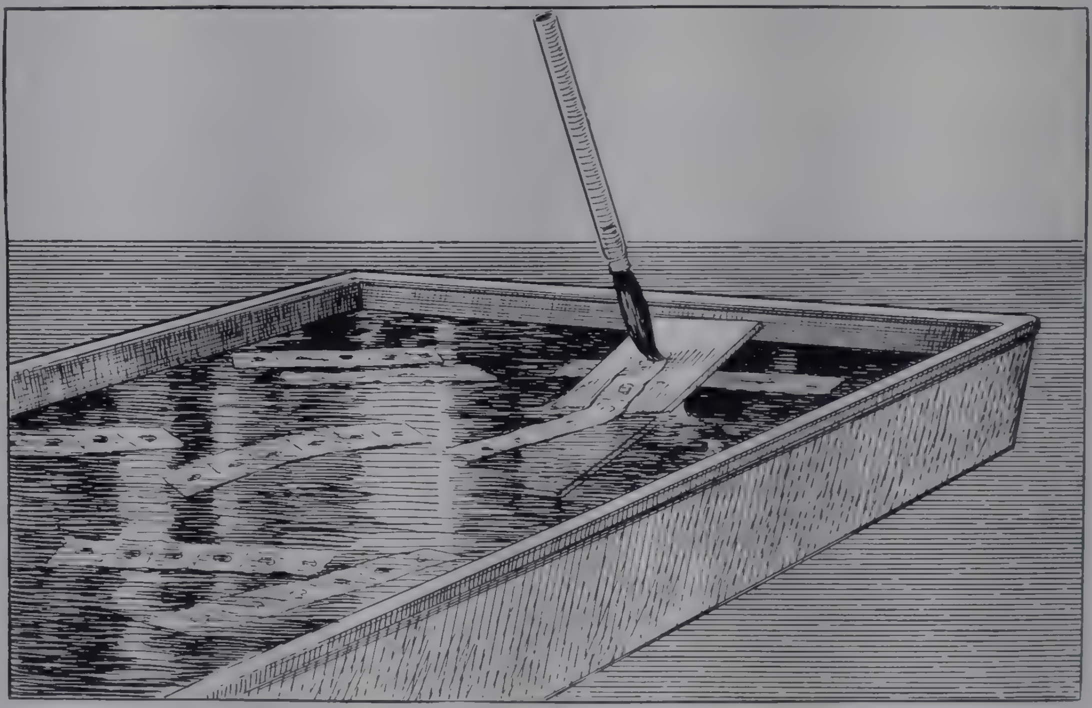

Fig. 623.—Placing paraffin sections on a glass slide.

9. Place on wooden block or pivot. Label back of block or pivot and retain the label throughout the process.

10. Cut tissue (see below). Cut it at right angles and parallel to the surface of the organ. The knife must be sharp. Cut the tissue 6 to 10 microns thick, or thinner if possible.

11. Rub Mayer's albumin fixative on the surfaces of glass slides. Sections are floated in warm water. Place tissue on slide.

12. Allow to stand in an upright position several hours to dry.

13. Stain, mount, and label.

Preferred Methods for Routine Paraffin Embedding

A. Rapid Method (Krajian).[1]—

Fix thin blocks of tissue in 10 per cent formaldehyde for 16 to 24 hours. In emergency cases, fix for one-half hour in paraffin oven at 56° C.

Place in acetone for 1 hour, using at least 20 times as much acetone as tissue.

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[1] Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, ed. 2, pp. 91 and 92. The C. V. Mosby Co., 1952.

Place in a second acetone for 1 hour, using the same quantity of acetone.

Place in benzine for 30 minutes.

Place in second benzine for 30 minutes.

Place in paraffin No. 1 in the paraffin oven for 1 hour.

Place in paraffin No. 2 in the paraffin oven for 1 hour.

Embed in the usual manner.

B. Slower Method (Krajian).—

Fix tissues as usual in 10 per cent formaldehyde.

Dehydrate in 70 per cent alcohol for 2 to 3 hours.

Dehydrate in 80 per cent alcohol for 2 to 3 hours.

Dehydrate in 95 per cent alcohol for 2 to 3 hours.

Dehydrate in a second 95 per cent alcohol for 2 to 3 hours.

Dehydrate in absolute alcohol for 2 to 3 hours.

Place in chloroform for 2 hours.

Place in the paraffin oven at 55° C. in a mixture of equal parts of chloroform and melted paraffin for 2 to 3 hours.

Place in pure paraffin in the paraffin oven at 55° C. for 2 to 3 hours.

Embed in the usual manner.

Krajian suggests that the acetone-benzine method be used for surgical tissues, while the longer method be reserved for sectioning tissues removed at autopsy.

C. A Benzine Method has been advocated by Krajian as follows:

1. Dehydrate tissues in 70 per cent alcohol 2-3 hours.
2. Dehydrate tissues in 80 per cent alcohol 2-3 hours.
3. Dehydrate tissues in 95 per cent alcohol 2-3 hours.
4. Dehydrate tissues in 95 per cent alcohol 2-3 hours.
5. Dehydrate tissues in absolute alcohol for 2-3 hours.
6. Benzine for 2-3 hours.
7. Benzine and melted paraffin, equal parts, in the paraffin oven at 55° C. for 2-3 hours (for the evaporation of benzine, leave the corks out of the bottle, otherwise the fumes may cause an explosion).
8. Pure melted paraffin in the paraffin oven at 55° C. for 2-3 hours.
9. Second paraffin bath in paraffin oven at 55° C. for 2-3 hours.

The Use of Dioxane or Isopropanol in the Process of Embedding

Dioxane.—The use of a series of graded alcohols, xylol, etc., described above in the preparation of animal tissues requires time and necessitates the constant attention of the worker. One of the bugbears in paraffin work is over-hardening of the object, making it difficult or impossible to section without tearing or cracking the microscopic sections.

For this purpose, an agent called ''dioxane'' (diethyl-dioxide) has been suggested and has proved remarkably efficient. It is miscible in all proportions with water and gives up water and methyl or ethyl alcohol to anhydrous calcium chloride; it is also miscible with melted paraffin. When one puts a piece of tissue into an alcoholic solution containing calcium chloride, the water is removed and the tissue is permeated with dioxane. Upon transfer of such a specimen to molten paraffin the dioxane is removed and the tissue infiltrated with paraffin ready for sectioning when cooled.

Technic.—

Use a vessel with the bottom covered with calcium chloride over which there is placed a fine wire net, on which the objects are placed.

Cover the vessel to prevent evaporation of the dioxane and absorption of water from the atmosphere.

After fixation, take the specimen from the fluid (Formalin) and place it directly into the dioxane and leave it there for 3 to 24 hours.

Transfer to the oven at 55-60° C. into a mixture of one-third dioxane and two-thirds paraffin and then into pure paraffin.

Keep the pure paraffin in a dish with gauze a short distance above the bottom as the dioxane tends to sink through it.

Isopropanol.—The use of isopropanol to replace graded alcohols, absolute alcohol, and xylol, except when reference is made to acid alcohol, is also recommended. It is very economical, being considerably cheaper than alcohol. Both isopropanol and dioxane are furnished by the Carbide and Carbon Chemical Corporation, 30 E. 42nd. St., New York, N. Y.

Cutting Paraffin-Embedded Sections*

Metal or wooden object holders are used to mount the paraffin block on the microtome. Metal holders are preferred. Warm the holder enough to melt the paraffin. Press the paraffin block onto the holder, then pass a hot needle around the edges of the base of the block to aid in firmly attaching it to the holder. If the blocks are large, it may be necessary to run a hot needle under the center of the block to aid in attaching it. After sectioning, remove the remainder of the block from the holder, label it with an adhesive or other type label, and store in the refrigerator.

When the object holder and paraffin block have cooled, proceed with the cutting. Set the microtome knife at a 90° angle to the direction of the cut (no slice angle) and tilt it to as little clearance as will give good sections (2 to 6°). Be sure the knife is *sharp* and shows no serrations.

Use the pencil type of camel's hair brush to handle the ribbon (unless single sections are to be made). Sometimes it is necessary to hold the first few sections onto the knife with gentle pressure of the brush until the ribbon forms. Set the automatic lift on the microtome for the desired thickness of the section and begin the cutting process. Raise the end of the ribbon with a dissecting needle over the brush. Withdraw this from the knife (with the left hand) as the ribbon lengthens, turning the microtome wheel with the right hand. Speed of cutting varies with the tissue. Proceed until the ribbon lengthens to a point where it is too long to handle. Place the ribbon lengths on smooth paper or in a shallow box until they are mounted on slides. Experience is required to cut serial sections without the loss of parts of the ribbon. Do not allow the brush to come in contact with the knife, or it will be spoiled. Do not use a dissecting needle in place of the brush, for, in case of accident of this type, the knife will be damaged.

One great inconvenience of paraffin sectioning, particularly in winter or if the atmosphere is dry, is the flying of sections and their sticking to parts of the microtome and other nearby objects due to static electrical charge generated by the friction of cutting. Raise the humidity by boiling water in the room or burning a Bunsen burner near the microtome to alleviate this difficulty. Sometimes it is necessary to ground the microtome to a nearby water pipe, using a wire or a chain.

After cutting the ribbon, it must usually be flattened before the sections may be attached firmly and evenly to the slide. Two methods are in general use:

1. Cut the ribbon into convenient lengths. Place on the slide containing albumin fixative. Add a few drops of water to the slide, and warm it gently until the sections expand to their full size.
2. Place the pieces of the ribbon onto warm water, allow to expand, and recover them by placing a slide containing a fixative under the tissues to pick them up.

Water used for "floating out" should be 5 to 7 degrees cooler than the melting point of the paraffin. Commercial wetting agents may be added to the water if desired. Do not stretch the section with needles, or by gravity by holding the slide perpendicular when draining off the excess water.

Tissue Flotation Bath[1]

The tissue flotation bath, with automatic temperature regulation, is an excellent and convenient apparatus for floating and spreading of tissue sections embedded in paraffin, preparatory to mounting. Bath temperatures can be controlled to within ± 1° C. in the range from room temperature to 60° C. It consists of a Pyrex glass tray, 10½ inches long by 6½ inches wide by 2 inches deep, and a low, insulated, Monel metal stand with flat copper top and built-in heating unit, thermoregulator, and pilot lamp. The top has raised edges, and a black oxidized finish, which aids in the inspection of translucent sections. The glass tray is readily removable for cleaning. The power consumption is 225 watts.

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*See O. W. Richards: The Effective Use and Proper Care of the Microtome, American Optical Co., Scientific Instruments Division, Buffalo, N. Y.

[1] The tissue flotation bath is obtainable from Arthur H. Thomas Company, West Washington Square, Philadelphia 5, Pa.

See Figs. 616 and 624.

Difficulties in Cutting

1. As block is being cut, there is a gritty feeling or a scratchy noise. Paraffin oven was too hot, or there is dirt in the paraffin.

2. Fixing agent may have crystallized and not washed out with water.

3. Sections jam on the knife: knife is dull, or paraffin is too soft, or paraffin has accumulated on the edge of the knife. Clean with xylol.

4. Paraffin rolls: knife is dull, or sections are too thick.

5. Objects crumble and drop out of the paraffin: insufficient paraffin penetration before embedding.

6. Ridges on specimen: nicks in knife or paraffin accumulated on edge of knife.

7. White patches near tissue in paraffin: caused by xylol carried into paraffin. Re-embed if near tissue. Cut off if near edge of block.

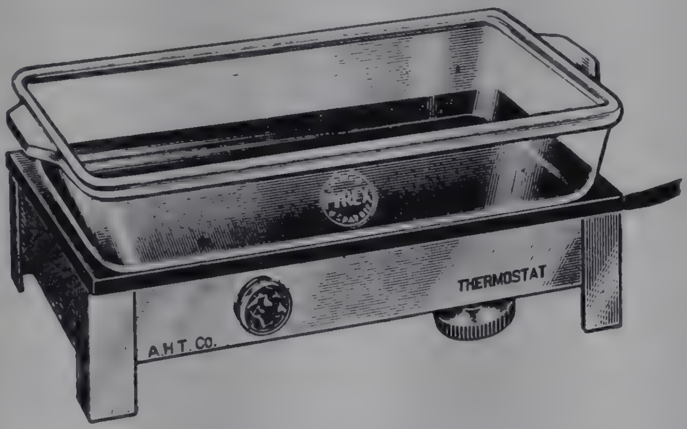

Fig. 624.—The tissue flotation bath. (Courtesy Arthur H. Thomas Co., Philadelphia.)

Difficulties Encountered in Cutting Ribbons

1. Ribbons fail to form. (a) Room is too cold or paraffin is too hard. Use softer (lower melting point) paraffin; warm knife slightly by blowing the breath on it, or immersing in *warm* (not hot) water; or place a desk lamp so that the light and heat fall on the knife and block. (b) Tilt the knife less. (c) Cut thinner sections. (d) Knife may be too dull—resharpen. (e) Dip block into a softer paraffin and trim so that a thin layer remains on the upper and lower edges of the block. (f) Unroll the section and hold it lightly against the knife with a camel's hair brush. If the first few sections can be held down, the ribbon will often form and follow.

2. Crooked ribbons. (a) When sections are wedge shaped the sides of the block are not trimmed parallel. (b) Edge of block may not be parallel to knife edge. (c) Try another part of the knife—sometimes irregularities of the knife edge cause crooked ribbons. (d) Or, the paraffin at one side of the block may be softer than at the other side, especially if the material has been re-embedded in a paraffin of different hardness—re-embed the material and stir the melted paraffin.

3. Sections compressed, wrinkled and jammed together. (a) The knife may be too dull. (b) Or, the room may be too warm; cool trimmed block and knife in very cold or ice water immediately before sectioning, or re-embed in a harder paraffin. (c) Tilt the knife more; the knife tilt may be too slight so facet bevel rubs over block. (d) The knife edge may be gummed with paraffin; wipe with finger or cotton moistened with xylol. (e) Soak block in water from an hour or two to overnight before cutting. (f) If cutting is done too rapidly, thin tissues will wrinkle.

4. Sections crumble and specimen may tear out. (a) Material incompletely dehydrated or not properly cleared. (b) When soft and mushy, material incompletely infiltrated —reinfiltrate and embed. (Salvage rarely possible if material was incompletely dehydrated.) (c) Alcohol not completely removed by clearing fluid. (d) Object too long in paraffin bath or paraffin too hot. (e) Subject hard and brittle due to clearing fluid. Try toluene in place of xylol or a mixture of toluene and cedar oil. (f) Try celloidin embedding or a rubber or asphalt mixture with paraffin for fragile material. (g) Try dioxane method for dehydrating.

5. Split ribbon or lengthwise scratches in ribbon. (a) If there are nicks in knife, resharpen or use another knife. (b) Use less tilt of knife so it will cut rather than scrape. (c) Knife edge may be dirty. (d) Object may be too large for paraffin method—use celloidin. (e) Hard particles in block may cause scratching. If there is dirt in the paraffin, filter or decant melted paraffin. There may be crystals from killing fluid (mercuric chloride); washing was insufficient. If calcareous or silicious particles are present, decalcify or desilicify.

6. Knife rings on up stroke and sections are scratched. (a) Change knife tilt to greater or lesser degree—tilt must be sufficient to clear facet bevel, but not enough to scrape instead of cut. (b) Material is too hard. Soak in water to soften; clearing may be at fault. (c) A thicker or wedge shaped knife may prevent springing of the edge when cutting. (d) Material may be too tough for paraffin method—try celloidin.

7. Sections lifted from knife on up stroke. (a) Increase knife tilt. (b) Room too warm or paraffin too soft—try harder paraffin or cooler room; or cool the block. (c) Knife may be dull—resharpen.

8. Sections stick to knife. (a) Knife edge dirty. (b) Increase knife tilt. (c) Try a sharper knife.

9. Undulations in the surface of the section. (a) Tighten all screws on knife and block holders and see that knife holder is clamped fast to microtome base. (b) Lessen excessive knife tilt to prevent vibration.

10. Scratching noise during cutting. (a) Material may be too hard, or small regions of material may be hard.

11. Sections fly and stick to parts of microtome or other nearby objects, due to static electricity formed from the friction of cutting. This usually occurs only in winter when the air is very dry. (a) Increase humidity of room by boiling water in an open pan, or burn a Bunsen burner in the room. (b) Ground microtome to a water pipe with a wire or a chain. (c) Ionize the air by an electrical method.

12. Sections vary in thickness or are skipped. (a) Knife not tilted enough to clear facet or bevel, or tilted too much, and tissue is compressed until the inevitable expansion gives a thick section. (b) Some of the clamping set screws on the block or knife holder are not tight or knife holder block not clamped firmly. (c) Microtome worn through lack of lubrication, or not in adjustment. (d) Very large blocks or blocks with hard regions may spring knife edge while sectioning—soak block in water to soften, use other methods for softening the material or embed in celloidin. The block will soak quicker if the paraffin is trimmed off one side to expose the tissue. Very little water is absorbed, but this process sometimes makes possible cutting hard or tough material.

TECHNIC OF EMBEDDING AND CUTTING CELLOIDIN SECTIONS[1]

Celloidin methods are used for large tissues and organs and for hard or delicate materials. The celloidin is not removed from the tissues and holds the delicate structures permanently together.

The material to be cut is embedded in one of the commercial celloidins (cellulose nitrate), the slow burning kinds being preferred. The disadvantages of the method are longer embedding time; serial sections cannot be cut, so that each section has to be handled individually; only dyes which do not stain celloidin may be used.

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[1] See O. W. Richards: The Effective Use and Proper Care of the Microtome, published by American Optical Co., Scientific Instruments Division, Buffalo, N. Y.

For materials not injured by moderate amounts of heat, the rapid process facilitates impregnation and preparation of the tissue. The slower, cold process takes more time and does not damage the tissue. It may not be possible to embed a hemisphere of a brain or a whole lung and harden it sufficiently for cutting in less than six months to one year.

The chief difficulty in celloidin sectioning arises from trying to cut improperly prepared material. Inadequately hardened blocks cannot be sectioned successfully. The blocks should be dense enough to cut at the required thickness. Unless the block is sufficiently hardened, the sections are uneven and distorted. If the block is too hard, irregularities are apt to occur. The surface of the block and the surface of the knife should be kept wet with 70% alcohol and as soon as the sections are removed they should be placed in alcohol.

The sliding microtome is the instrument of choice for cutting celloidin material, using a slice angle of 10 to 40°.

Tilt the knife a little more than for cutting with paraffin though the actual tilt will depend upon the hardness of the tissue. It is convenient to let large sections roll up on the knife. Then lift off of the knife and unroll into 70 per cent alcohol. Or remove the sections from the knife and lay a piece of filter paper on top of the section. It usually adheres to the filter paper and then both may be placed in a Stender dish. If the filter papers are numbered they may be kept in order. Or, stamp a number on the margin of the section with a commercial numbering machine as each one is cut.

The knife for sectioning celloidin-embedded tissue must be very sharp. Any irregularities in the edge will leave marks on the section. Keep the section and materials for embedding free from dust. Dust particles catching on the edge of the knife spoil the section, particularly when very thin sections are required. Remove silicious or calcareous material before sectioning. Do not allow the surface to dry. If it is necessary to stop sectioning for even a short time, cover the block with absorbent cotton and saturate with alcohol, or remove the block and place in alcohol or other storage liquid.

The so-called dry process has been developed to replace the method which requires keeping the block and knife wet with alcohol. After the tissue is embedded and hardened, soak the block in an oil (cedar oil) which lubricates the block during cutting. As the sections are removed from the knife, place them in the same oil. The oil-soaked block may be cut on a rotary microtome, as no slicing cut is required. This method is exceptionally good for making sections of difficult organs, such as the entire mammalian eye.

Special knife holders are available so that the knife can be turned to give a slicing stroke and may be used for cutting small celloidin blocks on a rotary microtome. The position of the block and knife is such, however, that it is difficult to keep the surfaces properly lubricated with alcohol so that the rotary microtome is not very satisfactory for this type of sectioning. If much sectioning in celloidin must be done on a rotary microtome, it is advisable to use the dry method which does not require a slicing stroke.

Celloidin embedded blocks are sometimes embedded in paraffin to hold them for sectioning. The size of the section which may be cut depends on the size of the microtome and the knife and the skill of the operator.

Celloidin Embedding, Method 1

For celloidin embedding we use a thin and a thick solution of celloidin. These two solutions are made as described below and placed in glass-stoppered, dark bottles to prevent the action of light and avoid evaporation of the ether. Entrance of air tends to cloud the celloidin and makes it unfit for use. If there is no need for hurry in the preparation of these specimens, the following technic may be changed so as to place the specimens one day in 1%, one day in 2%, one day in 4%, and several days in the 8 to 10% celloidin.

This method is very useful for certain types of tissue, particularly the eye.

1. Fix tissue in 10% Formalin.
2. 50% alcohol for twenty-four hours.
3. 70% alcohol for twenty-four hours.
4. 80% alcohol for twenty-four hours.
5. 95% alcohol for twenty-four hours.
6. Absolute alcohol for twenty-four hours.
7. Equal parts of absolute alcohol and ether for twenty-four hours.
8. Sections in thin celloidin 3% for twenty-four to forty-eight hours.
9. Place specimens in thick celloidin 6% to 12% for twenty-four to forty-eight hours.
10. The tissue is placed in thick celloidin in a small paper box and then under a bell jar in concentrated sulphuric acid in a dish until the specimen is firm. This removes all of the aqueous contents. If the embedded specimen shows any air spaces in the preparation, it is necessary to open these holes so as to let the celloidin enter better.
11. Remove sulphuric acid and put in chloroform. Let stand twenty-four hours.
12. Trim celloidin off around tissue, but do not trim too close.
13. Place celloidin block in equal parts of chloroform and cedarwood oil for twenty-four hours. Add a few grains of copper sulphate.
14. Mount on block of fiber. This can be carried out by placing a few drops of the thick celloidin on top of the block and then firmly pressing the specimen against it. Then place in cedarwood oil for two days.
15. Cut sections with sliding microtome, with the knife at an angle of 45° to the specimen. The knife should be constantly wet with alcohol while cutting, so that specimens will not be torn.
16. Place sections in 80% alcohol until ready to stain and until oil is removed.
17. Stain. Staining is carried out in just the same manner as paraffin sections are stained. (See page 2129 ff.)
18. Mount stained specimens on slide.

Celloidin Embedding, Method 2

There are various methods of using celloidin as an embedding material. Tissues for this type of embedding must be hardened and dehydrated for 24 hours in 80 per cent alcohol, followed by 24 hours in 95 per cent alcohol, and finally in absolute alcohol for an equal period. They are then soaked in a mixture of equal parts of alcohol and ether for 24 to 72 hours.

Impregnate in medium celloidin solution from 2 to 4 days, depending upon the thickness and consistency of the tissue.

Place in thick celloidin from 3 to 6 days. Blocks are then embedded in paper boxes or are mounted on vulcanized fiber. To harden the celloidin, leave the blocks exposed to the air until the celloidin mass has attained the right consistency. Then place the blocks in a dish containing pure chloroform, where they remain from 2 to 24 hours, and then place them in 80 per cent alcohol.

Directions for cutting celloidin sections are different from those for paraffin sectioning. First, a sliding microtome is used. The cutting movement should be slow and shearing. The surface of the knife should be kept constantly wet with 80 per cent alcohol from an overhanging drop bottle. Sections are removed from the knife, when cut, with a camel's hair brush and placed in 80 per cent alcohol. These sections are not fixed to the slides until they are ready for mounting. In other words, they are stained in watch glasses. It is not necessary to remove the celloidin to stain this type of section. During the process of staining, celloidin sections are likely to swell up and shrink. To flatten them, treat in equal parts of absolute alcohol and oil of cloves for 2 or 3 minutes before placing in xylol.

Thick and Thin Celloidin

Thick celloidin is made by dissolving 30 grams of dry celloidin in 450 c.c. of a mixture of equal parts of ether and absolute alcohol. Shake the receptacle several times a day for one week or longer and stir the sediment with a heavy glass rod at least once a day until solution is complete. Medium celloidin is made by diluting thick celloidin with an equal amount of ether and alcohol mixture. Thin celloidin is made as follows: Dry celloidin—12 grams; ether—200 c.c.; absolute alcohol—200 c.c. Shake until all celloidin is dissolved.

Difficulties Encountered in Handling Celloidin Embedded Material*

1. The chief difficulty comes from trying to cut improperly prepared material. Adequate impregnation of a large organ like a hemisphere of a brain may take a year. Improperly hardened blocks cannot be sectioned successfully. Pressure methods involving heat speed up the process and are available when warming will not injure the material.

2. Lengthwise scratches or splits in the section may be due to:

(a) Nicks in the knife. Use a different part of the knife or resharpen. (b) Particles of hard material in the block. Dust or dirt in the celloidin stock solution—let stand and use only upper portion after the particles have settled out or filter the stock solution. If calcareous or silicious deposits are present, decalcify or desilicify.

3. Specimen falls out of section, is mushy and soft. (a) Dehydration was incomplete. (b) Infiltration was incomplete—refilter, re-embed, and harden. (c) Harden block if too soft, in chloroform, or a mixture of equal parts of 95% alcohol and glycerin.

4. Variation in thickness of sections. (a) Loose screws on knife or block holders—tighten all set screws. (b) Knife holder depressed or raised by the hand while sectioning—hold knife block so as not to move it vertically while cutting. (c) Knife not tilted enough to clear facet of cutting bevel. (d) Knife too dull. (e) Microtome worn and out of adjustment. (f) Material not hardened properly. (g) Slight drying of block between sections.

STAINING TECHNIC FOR PARAFFIN AND CELLOIDIN SECTIONS

Staining Technic for Paraffin Sections

Method No. 1. Recommended Procedure[1]

This method is an adaptation of the staining technic devised by Krajian[2] for staining frozen sections. It gives excellent results. With this method sections are seldom lost during the staining process.

Prepare paraffin sections in the usual manner. Cut them as thin as possible, and mount on a glass slide without fixative. If a fixative is considered necessary, use Mayer albumin fixative. Allow the tissues to dry on the slide. Carry out the process from here in staining jars.

Place slides in xylol No. 1 in a Koplin jar for five minutes.

Transfer to xylol No. 2 in a staining jar for another five minutes to insure removal of paraffin from sections. If necessary, use a third xylol bath.

Place slides in anhydrous isopropanol or absolute alcohol to remove the xylol. This usually requires five minutes.

Blot twice with several thicknesses of Whatman No. 1 filter paper.

Immediately dip twice in thin celloidin (page 2105). Keep the slide perpendicular while dipping, and dip quickly, but not suddenly. Do not turn the slide while dipping in and out. Blow gently over the slide while holding it in a perpendicular position. Continue the gentle blowing until the celloidin is dry. Wipe the celloidin off the back of the slide. The slide is now ready to be stained.

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*We are indebted to Dr. O. W. Richards for use of this material from his pamphlet "The Effective Use and Proper Care of the Microtome," American Optical Co., Scientific Instruments Division, Buffalo, N. Y.

[1] Gradwohl, R. B. H., and Erskine, A. G.: Lab. Digest **10**: Nov., 1946.

[2] Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, St. Louis, 1952, The C. V. Mosby Co.

Carry out the staining technic from this point as for frozen sections, method of Krajian, page 2105.

Method No. 2 for Staining Paraffin Sections

Reagents.—

The **various alcohols** are described on page 2120.

Delafield's Hematoxylin.—

Make a saturated solution of ammonium alum in distilled water.
Dissolve 4 gm. hematoxylin crystals
in 25 c.c. 95% alcohol
Add 400 c.c. saturated solution of ammonium alum.

Place in a Mason jar. Place the cover on lightly, and put in the light and air for one or two weeks.

Add 100 c.c. methyl alcohol (acetone-free)
and 100 c.c. glycerin.

Keep in a bottle exposed to the sunlight until ready for use. It usually requires at least one month for the hematoxylin to ripen.

Harris' Hematoxylin.—See page 2105.

Boehmer's Hematoxylin.—See page 2135.

Ehrlich's Hematoxylin.—

1. Hematoxylin	2 gm.
Absolute alcohol	10 c.c.
Glacial acetic acid	10 c.c.

Dissolve the hematoxylin in the mixture of alcohol and acetic acid.

2. Add 90 c.c. absolute alcohol to
100 c.c. glycerin, and mix
3. Add the hematoxylin solution to the alcohol-glycerin mixture.
4. Dissolve 10 gm. of potassium alum
in 100 c.c. distilled water with heat.
Cool.
5. Add the alum solution to the hematoxylin-alcohol-acetic acid-glycerin mixture, stirring constantly.
6. Expose the solution to light and air to "ripen" for 4 to 6 weeks. If the solution stands in a warm place and is shaken occasionally, oxidation is facilitated, and the ripening process is aided materially. The ripe solution should have the color of port wine.

Mallory's Phosphotungstic Acid Hematoxylin.—See page 2155.

Acid Alcohol.—Add 1 c.c. of chemically pure concentrated hydrochloric acid to enough 70% alcohol to make 100 c.c. total.

Alkaline Alcohol.—

(a) Dissolve 2 grams of sodium bicarbonate in 100 c.c. of 70% alcohol,

or

(b) Add 1 c.c. of concentrated ammonium hydroxide to enough 70% alcohol to make 100 c.c. total.

0.5% Alcoholic Eosin.—

Dissolve 500 mg. of eosin in 100 c.c. 95% alcohol.

Technic.—

All solutions are put in Koplin jars, or large staining jars, Figs. 611 and 625, and the jars arranged in the proper sequence. When transferring slides from one solution to another, catch the excess solution on a cloth as it drains off.

DO NOT LET THE TISSUE DRY DURING THE STAINING PROCESS.

Immerse in xylol five minutes to dissolve the paraffin.

Absolute alcohol	1 minute.
95% alcohol	1 minute.
80% alcohol	1 minute.

70% alcohol -- 1 minute.
50% alcohol -- 1 minute.
Do not make changes too rapidly, or tissue may fall off the slide.
Tap water -- 1 minute.

Hematoxylin. (Delafield's, Harris,' or Ehrlich's. Harris' is preferred. Hematoxylin accomplishes the basic staining of the nuclei—five minutes or longer. If tissue is not the correct shade of purplish blue, replace in the hematoxylin. Look at the section under the microscope to determine the correct intensity of the stain.

Tap water until no more color comes off.

50% alcohol. Wash. Just put the slide in and out.

Acid alcohol. (1% hydrochloric acid in 70% alcohol.) Dip the slide in the jar, then hold it above until the tissue is salmon pink. DO NOT DECOLORIZE TOO LONG.

Alkaline alcohol until blue. (2% sodium bicarbonate in 70% alcohol or 1% ammonium hydroxide in 70% alcohol.)

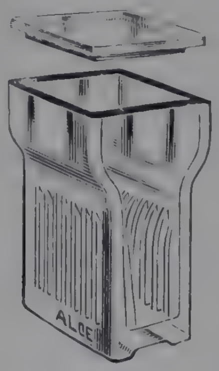

Fig. 625.—Wide top staining jar. (Courtesy A. S. Aloe Co., St. Louis, Mo.)

Look at tissue under microscope to ascertain whether nuclei are sufficiently blue. If they are not replace the slide in acid alcohol, then 50% alcohol, then water, then hematoxylin, then continue on from this point.

80% alcohol -- 1 minute.
95% alcohol -- 1 minute.

0.5% alcoholic (95%) eosin Y. Dip in. About ½ minute or longer.

Wash in 95% alcohol, not too long as the eosin decolorizes.

100% alcohol, not too long otherwise eosin may disappear.

Xylol until clear, usually about 3 to 10 or 15 minutes. Look at the tissue under the microscope. If it is not red enough, go through the alcohols back to the eosin; if not blue enough, go back through the process to the hematoxylin.

Put a drop or two of gum damar dissolved in xylol over the tissue.

Mount with a cover glass without air bubbles.

Label.

Points to Be Remembered.—

Always wipe off excess of solution between each Koplin jar.

In embedding and in preparation for embedding, there should be about 5 times as much solution as tissue.

Solutions and glassware should be clean.

Tissues can remain longer than one hour, if necessary, in any of the alcohols except the absolute.

We suggest the use of Arthur H. Thomas paraffin, melting point 56°-58° C., Tissuemat, or Histowax (see page 2121) for embedding.

Keep the oven at 58°-60° C. (2° higher than the melting point of the paraffin).

If while cutting you obtain no ribbons, put powder on the blades.

Keep the blade at an angle of 45 degrees.

When using the fixative, use a drop of Mayer's and rub. It is immaterial if it dries; do not rub dry. Place tissues on it. Run distilled water under the tissue and bear down slowly until all wrinkles are removed from the paraffin. Heat gently if necessary. DO NOT MELT THE PARAFFIN. Drain off the water. Mark the slide, set it up to dry (two hours).

Slides may be placed in the bacterial incubator to hasten drying.

If a pan of water is used to float the tissues, the water must be hot enough to spread the tissues, but not hot enough to melt them.

Rub off excess fixative.

Never put tissue in the oven when it is in xylol.

Slides must be cleaned before presenting to pathologist. Be sure a history accompanies each specimen.

Difficulties Encountered in Staining

A. If objects do not remain on the slide:

1. Slide may have been dirty or oily.
2. Albumin fixative is too old. (If too old, it loses its clear color. Or it may have an odor. Make it in 1 oz. quantities. This will last two or three months.)
3. Slides might have been carried through the alcohols too fast.
4. Dehydration of the tissue through alcohol may have been too rapid. (If tissue is too small, use 60% alcohol after the 50%.)
5. Paraffin section not thoroughly spread on slide when mounted.

B. If objects do not stain:

1. Stain is at fault. Hematoxylin must ripen. Do not use it too soon after preparation.
2. Stain has deteriorated.
3. Paraffin had not been thoroughly removed from the slide. Leave longer in xylol. It can be run back.
4. Early fixation in alcohols before paraffin was not correct; nothing can be done to remedy this.
5. Fixing agent not thoroughly washed out of tissue before immersing in alcohols.

C. If section is not clear under microscope:

1. Xylol should be replenished.
2. There was water in the absolute alcohol.
3. Moisture on the cover slip.
4. Too much egg albumin on slide.
5. All the acid after acid alcohol decolorization was not removed.
6. A film from the alkaline alcohol was carried along.

To Remedy:

Put in Koplin jar of xylol to dissolve gum damar. Run back through the processes to the jar where the fault lay and use fresh solutions. Then restain.

If the tissue is thoroughly adherent to the slide, it can be taken back several times without danger.

Additional Pointers

When mounting tissues on slides, make several at once.

Thick sections come off the slide.

Occasionally you cannot cut a tissue less than 10 microns thick.

If the tissue is likely to come off:

Put slide in xylol to dissolve paraffin.

Dip in solution of ¾ gm. celloidin in 100 c.c. ether-alcohol (equal parts).

This places a slight film over the slide and does not interfere with the penetration of stain (except carmine).

After cover glass is on, if gum damar creeps out, rub off with xylol cloth.

Keep knives sharp.

If tissue is not embedded well, melt the paraffin in oven and re-embed it.

Rapid Staining Method, Employing Hematoxylin and Eosinol

Krajian[1] has offered a method of hematoxylin and eosin staining which affords a margin of safety between excessive extraction of eosin and complete elimination of water. This method overcomes the difficulty of loss of color of eosin and also has the advantage of saving alcohol and time.

The principle of the modification consists in the dehydration of the section before counterstaining and the application of eosin as a solution in carbol-xylol. Then, since the tissue passes afterward through xylol only, there is no opportunity for decolorization. Alcohol is saved because dehydration can be commenced in the stronger alcohol, discarding the lower gradations. The solution of eosin in carbol-xylol, which constitutes the principal element of modification, may be called, for convenience, ''Eosinol.''

Eosinol Preparation.—Dissolve 5 gm. of yellowish water-soluble eosin in 10 c.c. of distilled water. Precipitate it by adding 10 c.c. of glacial acetic acid, 2 c.c. concentrated hydrochloric acid, and mix with a glass rod. Incubate the resulting coagulum at 56° C. for 12-16 hours, or until all the water has evaporated.

Dissolve this dehydrated acid eosin in 10 c.c. of absolute alcohol and 20 c.c. of acetone, stirring with a glass rod for several minutes. Tightly cover and place in a paraffin oven for 2 hours. Let the undissolved portion, which is to be discarded, settle to the bottom of container (about 10 minutes). Remove clear portion with a clean dry pipette; add it to 1,500 c.c. of carbol-xylol (one part pure melted phenol crystals in three parts of neutral xylol). Some precipitate will form, which will settle to the bottom of the container. The clear portion is ''Eosinol.'' The solution keeps indefinitely. All glassware must be dry.

Owing to the nonuniformity of staining power of the various brands of powdered eosin, it may be necessary to standardize the solution by staining control sections and adjusting the strength of the eosinol by reducing or increasing the amount of carbol-xylol.

The Staining Method.—1. Prepare microscopic sections by one of standard sectioning methods (frozen, paraffin, celloidin) and stain according to directions on page 2105.

Paraffin Tissue Preparation

Method of Hektoen

Fix tissue in following solution:

Saturated solution mercuric chloride	3 parts
95% alcohol	1 part

(Or use Zenker's solution.)

Tissue can remain in mercury and alcohol solution for an indefinite period of time, but not over twelve hours in other solutions. After tissue has been fixed, place and tie in gauze, and wash under running water from two to twelve hours.

Dehydrate by placing in 80%-85% alcohol for one hour.

Remove and place in absolute alcohol or acetone for one hour.

Pour off and repeat same for another hour.

Remove, and place in cedarwood oil for about twelve hours.

Remove and dry oil with blotting paper.

Infiltrate in paraffin four hours in the following manner:

Use soft paraffin. Place 4 different receptacles half filled with paraffin in paraffin oven and keep temperature between 52° and 54° C. to keep paraffin melted, and place tissue in each jar for one hour.

Remove and embed by placing tissue in pan containing

Hard paraffin	3 parts
Soft paraffin	1 part

Place in ice box until paraffin is hard, then cut and mount on hardwood blocks. After specimens are blocked, place in ice box until ready to section.

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[1] Lab. Digest 1: No. 10, 1938.

Cut with microtome (have knife very cold) and place sections in dish of warm water to straighten ribbon formation before placing on slide.

Use Mayer's fixative. (Page 2115.)

Smear slide slightly with above mixture.

Place ribbon containing 3 to 5 sections of tissue on slide. Place in warm oven overnight to dry. Place in oven about 4 minutes at 80° C.

Stain as follows:

Xylol, five minutes or longer to dissolve paraffin.
2% iodine to remove mercury.
95% alcohol to remove iodine.
Wash under running water two minutes.
Hematoxylin, two minutes.
Running water, five minutes.
2%hydrochloric acid until salmon color.
Wash in running water.
1% solution ammonium hydroxide until blue.
Wash in running water.
1% solution of eosin in distilled water, 1 to 2 minutes, or longer, if desired.

Wash. Examine the tissues under the microscope after each step to determine if the correct color is obtained.

95% alcohol, two to three minutes.
Absolute alcohol and xylol, three to five minutes.
or
Acetone and xylol for three to five minutes.
Xylol to clear, five to ten minutes.
Remove, blot, dry, and mount in Canada balsam.

Liver Biopsy

Liver biopsy is discussed on pages 1228 ff.

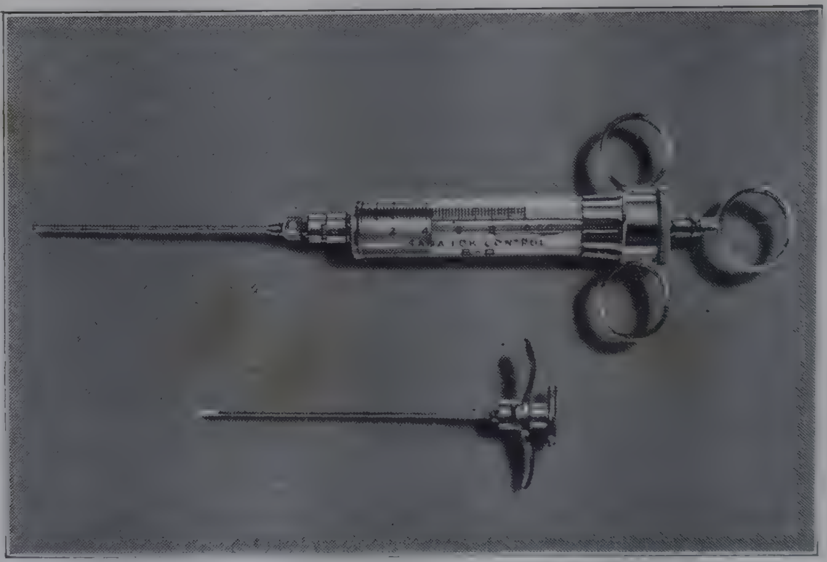

Fig. 626.—B. D. Feder-Wrenn biopsy needle and B. D. biopsy syringe. (Courtesy Becton, Dickinson and Co., Rutherford, N. Y.)

Procuring Tissue From Biopsy With the Feder-Wrenn Needle

The Feder-Wrenn tissue biopsy needle has been successfully used in surgical biopsy work. The method of obtaining the specimen is as follows:

Apply an antiseptic to the skin, then inject a local anesthesia, and insert the stilet and cannula into the skin and into the border of the tumor.

Remove the stilet and introduce the cannula several millimeters further. In this way a small plug of tissue is imprisoned within the bore of the instrument.

Now attach a No. 10SLB biopsy syringe and obtain suction by withdrawing the plunger until the lock catches in place. Due to this aspiration, tissue is drawn into the cannula.

Using the syringe as a lever, apply a gentle rotary motion to break the impaled specimen loose at its base.

Now withdraw the needle from the tissue, release the lock, and use sterile saline solution to flush free adhering particles.

This needle, No. 451LNR, 13G., 3″, may be obtained from Becton, Dickinson, and Company, Rutherford, N. J.

Technic of Making Large Sections of Organs as Originated by Christeller

A specially constructed sliding microtome made by Leitz, with a large freezing table, is used.

Slides are photographic plates 9 × 12 cm. Cover glasses slightly smaller, also furnished by Leitz, are used. These are approximately 8½ × 11 cm. Staining dishes are rectangular-shaped museum jars. There should be about 12 of these. Tissues should be hardened in Formalin, 10%.

Place a moistened piece of good grade lens paper on the microtome block.

Place the tissue to be cut on this, and press down.

Cover with the freezing hood, and freeze slowly.

When the tissue is frozen, remove hood, and cut.

Moisten the fingers. Cut one slide at a time, lifting the slide with the fingers as you cut. Be careful not to cut your fingers during this process.

Place the sections in water with fingers after removing from blade.

Place sections on plates with bent glass rod with blunt end. This is not easy. It takes several days' practice to become efficient in this process.

There must not be a single bent place on the section.

Dehydrate with absolute alcohol. Use a large quantity of alcohol.

Press out all air bubbles with rod.

Dehydrate three or four times.

Cover with a thin layer of very thin celloidin. Let it be thin enough when placed on the back of the hand to make an extremely thin coating.

Run excess back into bottle.

(Make up this celloidin from stock solution, using 2 c.c. to 100 c.c. of equal parts of alcohol and ether.)

Wait until it is almost air dried, but *not* dried.

Solutions for staining are placed in the museum jars (11 × 15 cm.).

Lay the specimens in water directly.

Place in Boehmer hematoxylin (below) for three to four minutes.

Distilled water.

Hydrochloric acid-alcohol, 1 c.c. hydrochloric acid to 100 c.c. 70% alcohol.

Distilled water. Repeat in fresh water to remove acid. Section becomes blue.

Eosin. Aqueous solution.

Water, distilled.

Alcohol 70%, one minute.

Alcohol 95%, one minute. Do not use absolute alcohol, since this re-dissolves the celloidin.

Terpene oil (Kahlbaum) or carbol-xylol, one minute.

Xylol.

Canada balsam. Put balsam in middle of plate and slightly bend the cover glass to middle, let it down on both sides and press, put weight on it overnight.

Label.

Boehmer's Hematoxylin.—

Solution I.—	Hematoxylin crystals	1 gram
	Absolute alcohol	12 c.c.
Solution II.—	Alum	20 grams
	Warm distilled water	200 c.c.

Filter when cool.

After twenty-four hours, mix both solutions. Place in a wide-mouth bottle and expose to air for eight days.

Filter through filter paper.

Preparation of Small Pieces of Skin Tissue[1]

Bouin's Solution.—

Saturated aqueous picric acid	300 c.c.
Formalin (40%)	100 c.c.
Glacial acetic acid	20 c.c.

50% Alcohol, 70% Alcohol, 80% Alcohol.—

See page 2120.

Technic.—

Fix tissues in Bouin's solution overnight. Twenty-four hours is the maximum fixing time.

9:00 A.M. Wash in 50% alcohol.

9:00 A.M. Wash in 70% alcohol containing 3 drops of a saturated solution of lithium carbonate.

12:00 Noon. Put in 70% alcohol. The tissue can be left over the week-end in this solution.

3:00 P.M. Place tissues in 80% alcohol.

5:00 P.M. Place tissues in 95% alcohol and leave until the following day.

Next day, 9:00 A.M. Place in absolute alcohol.

10:30 A.M. Place in amyl acetate.

4:00 P.M. Place in paraffin in the paraffin oven.

5:00 P.M. Pour off the paraffin and leave the tissues outside of the paraffin oven. Never leave tissues in oven overnight. Never re-use any paraffin.

Next day, 9:00 A.M. Put in second paraffin.

12:00 Noon. Put in third paraffin.

2:00 P.M. Embed in paraffin.

Cut and stain with routine hematoxylin and eosin stain (pages 2105 ff.).

Amyl acetate can be used for clearing Formalin-fixed tissues as well as those fixed in Bouin's fixative.

If the tissue is fixed in Formalin, first wash in water from 9:00 A.M. until 10:30 A.M. Then place in 50% alcohol and then in 70% alcohol until 12:00 noon. Follow the technic for Bouin-fixed tissue from this point.

This method is especially recommended to make satisfactory sections of very small pieces of skin, biopsy, etc.

Preparation of Serial Slices of the Breast

Method of Ingleby and Holly[2]

In relation to diagnosis of disease of the breast, it is of paramount necessity to examine as large portions of this organ as possible in order to come to the proper conclusion concerning the pathologic anatomy. In other words, the organs as a whole must first be studied and later any special area should be minutely examined.

Technic.—

Specimen is brought from the operating room or autopsy table.

Suspend it in a jar of formaldehyde (4 per cent) in order to fix it with the normal contour preserved as nearly as possible. Suspending the specimen can be accomplished by means of a gauze sling.

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[1] Barnard Free Skin and Cancer Hospital of St. Louis, Mo., Lab. Digest 9: No. 10, March, 1946.

[2] Journal of Technical Methods and Bulletin of the International Association of Medical Museums, No. XIX, 1939.

Place the jar containing the breast in the refrigerator room, and leave it there for at least 24 hours.

Remove from the solution, wrap in a wet cloth, and freeze. Freeze rapidly. Use a commercial ice cream freezer for the purpose or place in a freezing unit.

Ingleby and Holly recommend the slicer made by the American Slicing Machine Co. This machine cuts even slices down to about 0.5 mm. in thickness. With this slicer, freezing of the tissue is not absolutely necessary; however, better results are obtained if the specimen is frozen before slicing, especially if the specimen is of soft or uneven consistency, such as fatty breast where skin is present.

Stack sections in order. They may be put back into formaldehyde for further fixation, or they may be tagged and numbered at once. Some sections are difficult to handle. Receive these in a bowl of water and float onto numbered pieces of paper, and stack. In this way, sections can be kept perfectly flat during further fixation.

Fix for a few hours, then wash one hour or more in running water, rinse in distilled water, and place in a single layer in the following filtered solution:

Harris hematoxylin	5 c.c.
Distilled water	100 c.c.

Stain for 24 hours, turning once during this time to insure even staining.

Differentiate in acid alcohol.

Wash until blue.

Place in 70% alcohol	1 to 2 hours,
95% alcohol	6 to 8 hours (two changes),
absolute alcohol	2 to 4 hours (two changes),
benzine	2 to 4 hours (two changes).

Store in mineral oil until required for use. Then transfer to oil of wintergreen 2 parts and benzol benzoate 1 part for about an hour. They are then ready to be examined under the dissecting microscope.

An alternative stain is Masson's iron hematoxylin. This gives excellent results where material to be stained is not too dense. It is the method of choice if photography is contemplated.

Sections must be returned to the mineral oil after study. Oil of wintergreen is an excellent clearing agent, but it extracts the hematoxylin and the sections fade rapidly. In some cases sections may be examined in mineral oil without clearing. Occasionally, especially with the ordinary microscope, mineral oil gives better results than the more brilliant oil of wintergreen.

Advantages of the Method.—

1. It is possible to examine grossly every part of the tissue. In this way small lesions are not missed and if it is not desired to stain whole sections, suitable pieces for paraffin blocks are easily picked out.
2. A three-dimensional view is obtained and structural relations are beautifully shown; no lesion of any importance can be missed.
3. The method is a rapid one. A whole breast could be ready for examination in five days or less.
4. Paraffin sections can be made at any time from any part should higher magnification be desirable.

SPECIAL METHODS OF STAINING

Feder Modification of the Rhamy Stain[1]

The stain developed by Feder for use in connection with frozen-section technic of Rhamy consists of

Toluidine blue	0.8 gm.
Basic fuchsin	0.3 gm.
Eosin Y	0.4 gm.
Alcohol, 95%	33.0 c.c.
Distilled water	77.0 c.c.

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[1] J. M. Feder: Essentials of Applied Medical Laboratory Technic, Charlotte, N. C., 1940, Charlotte Medical Press, p. 217.

Weigh the dyes and dissolve them in alcohol. Add the water and allow to stand overnight. After thorough agitation to insure solution, filter. Keep the stain in a dropping bottle ready for use.

Set up microtome and knife. Adjust micro gauge to about 7 microns. Do not strive for a very thin section. A 10-micron section showing the structure in its continuity is much better than one of 5 microns that is distorted.

Obtain two glass bowls of about half a pint capacity and about 6 inches in diameter. Fill one of these with 4 per cent Formalin. Fill the other bowl with tap water.

Obtain the desired piece of tissue. It should not be more than the thickness of a quarter-dollar and not over a third of its size. If the material has not been Formalin-fixed, drop it in a dish of 10 per cent Formalin, heat to the boiling point and allow it to remain there for one or two minutes. Wash in water for an instant and transfer to the freezing block of the microtome. The water adhering to the tissue will be sufficient to fix it to the freezing disk. Turn on the CO_2 from the tank and, using the control on the microtome, turn the gas on and off several times until the tissue is frozen half-way through.

With the adjusting wheel, run the tissue up until the knife is in line with a point just above the zone of freezing. Cut here. Wet the knife with water and continue to cut sections until the frozen part is reached. Pick the sections up on the finger tip and transfer them to the bowl containing the 4 per cent Formalin solution. When enough satisfactory sections have been cut, with the aid of a camel's-hair brush, cut to a fine point at the tip, transfer one to a slide. (Some use a glass rod to pick up sections and transfer them to a dish of stain and from this to water but Feder finds that smoother sections can be made by staining them flattened on a slide.)

Cover section with the special stain described, allow it to remain for 20 seconds, then transfer to a bowl containing water by dipping slide into it. Paddle section around for a few seconds with the end of slide to rid it of excess stain, and remount.

Place a drop of mounting fluid on a coverglass (this mounting fluid for frozen sections consists of equal parts of 95 per cent alcohol, glycerin, and distilled water).

Wipe excess fluid from about section and back of slide, gently apply cover glass containing mounting fluid, and the section is ready for examination. The staining reaction closely approximates that of the routine hematoxylin-eosin method commonly used; therefore, the examiner does not have to become accustomed to new color combinations.

Twenty-Minute Method of Staining Treponema Pallidum in Frozen Sections—Krajian

(For the Diagnosis of Syphilis)

In 1939, Krajian described his excellent and practical method of demonstration of the *Treponema pallidum* in biopsy material particularly designed to facilitate the diagnosis of primary syphilis. It is the best and most simple method yet devised. It gives a very clear-cut picture of the organism of syphilis, and that, too, in many instances where dark field examinations have failed to show the organism.

It might be mentioned that the dark field method is positive in about 90 per cent of cases in the first week of the lesion, 50 per cent in the fifth week. The morphology of the spirochetes found on the surface of mucosal lesions, by dark field and smear methods, may be of less significance than those found deep in the tissues of biopsy sections, because here their recognition is not affected by saprophytic organisms and free oxygen.

The twenty-minute method of Krajian follows:[1, 2]

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[1] Krajian, A. A.: Am. J. Syph. 23: 617, 1939.

[2] Krajian, A. A. and Gradwohl, R. B. H.: ed. 2, The C. V. Mosby Co., 1952.

Reagents.—

Primary Solution

Uranium nitrate	1 gm.
Formic acid, c.p., 85%	3 c.c.
Glycerin, c.p.	5 c.c.
Acetone, c.p.	10 c.c.
Alcohol, 95%	10 c.c.

10% Silver Nitrate Solution.—

Dissolve 10 gm. of silver nitrate, c.p., in enough distilled water to make 100 c.c. of solution. The 1% solution is made from this.

1% Silver Nitrate Solution.—

The 10% silver nitrate solution will keep for months. Dilute it just before using by adding 3 c.c. of 10% silver nitrate solution to enough distilled water to make 30 c.c. of solution. This solution may be used a maximum of three times during the course of the same day. Do not use it if it has a brownish cast.

Developing Solution.—

Hydroquinone	0.31 gm.
Sodium sulphite	0.1 gm.
Acetone	2.5 c.c.
Formaldehyde, 40%	2.5 c.c.
Pyridine	2.5 c.c.
Saturated solution of gum mastic in 100% alcohol	2.5 c.c.
Distilled water	15.0 c.c.

Dissolve the hydroquinone in formaldehyde and acetone, then dissolve the sodium sulphite in this solution. Then add the other reagents and mix. Finally add the gum mastic. With the addition of the water, the mixture assumes a smooth creamy appearance.

This solution may be used a maximum of three times during a day's work, but should preferably be used in small quantities and used only once. The stock solution, if kept out of the light, will remain good for at least one or two weeks. Keep out of the cold to prevent separation of the gum.

Saturated Alcoholic Solution of Gum Mastic.—

Saturate 25 gm. of gum mastic with 35 c.c. of absolute alcohol. Shake several times a day for 3 to 5 days until clear. Allow to precipitate and use only the clear supernatant portion. Clarification can be hastened by centrifuging.

Technic of Staining.—

If the tissue has not been previously fixed in 10% Formalin, boil a small piece of biopsy material in 10% Formalin in a Pyrex test tube, agitating it for one minute. Or, drop blocks of tissue about 5 mm. thick in a 10% Formalin solution at 67° C., and place in an oven at 67° C. for 10 minutes.

Freeze and cut immediately. Cut frozen sections about 7 to 10 microns. Wash in distilled water.

Place the *Primary Solution* and the *Developing Solutions* in small Stender dishes in the paraffin oven at 60° C. to keep them warm. Have three small Stender dishes containing distilled water. Place about 5 to 10 c.c. of 95% alcohol in another Stender dish. In another Stender dish, place 5 c.c. of 95% alcohol and add about 3 drops of saturated solution of gum mastic solution from the end of a small glass lifter. The 1% silver nitrate solution should be in a 250 c.c. Pyrex beaker.

Place the tissue in the Primary Solution at 56 to 60° C. for 5 minutes. This can be kept in the paraffin oven to maintain its temperature.

Rinse in distilled water. The tissue should open up and float on the water in the Stender dish.

Pick up with the glass lifter, and keeping it on the lifter, pass three times gently but quickly through the weak gum mastic mixture.

Place in another Stender dish of distilled water. The tissue should open up and float.

Transfer section to a wide-mouth 250 c.c. Pyrex beaker containing 30 c.c. of 1% silver nitrate solution. The beaker should be on an asbestos board over a low flame and under a 60-watt electric light. The light should be about 4 feet above the beaker. Heat the silver nitrate solution under the electric light until bubbles form, keeping the temperature between 70 and 73° C. for seven minutes.

Without washing, carry individual sections with the glass rod lifter to the warm developer solution. This solution should be kept hot over the burner. Alternately lift in and out of the solution, exposing every part of the tissue to the electric light. When replacing each time, be sure the section opens out on the developer solution. This cannot be accomplished unless the solution is quite warm. Repeat this alternate exposing and developing process about eight times. The tissue turns brown.

Transfer to the 95% alcohol in a Stender dish to remove excess gum mastic.

Transfer to a Stender dish containing fresh distilled water. The tissue should straighten out here. If it does not, replace for a few seconds in the alcohol, then replace in the distilled water.

Place once more in the cooled silver solution and expose to the electric light for an additional 10 to 30 seconds. This will cause the spirochetes to assume a good black color.

Transfer to a large bowl of distilled water. **Do not use tap water.**

Transfer to a glass slide. Dehydrate by dropping a few drops of isopropanol over the surface, blow gently at first, then blow a little harder to remove water.

Blot under several thicknesses of No. 1 Whatman filter paper, place isopropanol over the slide, and allow to dehydrate for at least one minute. Again blot with Whatman No. 1 filter paper after draining off the excess isopropanol.

Dip once in thin celloidin (page 2105). Wipe the bottom of the slide dry. Blow over the surface of the celloidin until it is thoroughly dry.

Place the slide on a staining dish and cover with isopropanol for sixty seconds to dehydrate.

Place in xylol for several minutes. Place in a second xylol to clear. Clean excess celloidin from around the tissue and mount with gum damar. Examine with the oil immersion objective.

Pointers.—

The spirochetes should stain black against a light brown background. If they are brown either the second silvering process was not carried out properly or the developing solution was too cold. Or the developer might have separated, in which case make fresh developer.

When the gum mastic separates out of the developer, make a fresh solution. The Primary Solution keeps indefinitely.

If some of the spirochetes are brown and some are black, you have not spread the sections properly in the developing solution. Be sure the solution is hot so that the tissues will open properly.

If some portions of the tissue do not show spirochetes and you feel that they must be there, it may be due to improper fixation of the tissue. Unfixed portions of the section would not permit staining of the spirochetes.

When gum mastic is old or is in solution, it deteriorates.

If the lesion has been covered or treated with Mercurochrome or other chemicals the staining will be affected.

Dirty glassware will cause precipitates to appear in the section. Clean with sulphuric acid dichromate cleaning solution.

If the tissue suddenly shrinks when it is removed from the silver nitrate solution and placed in the water, it may be due to the fact that the water is not really distilled water, or the silver nitrate for the second silvering process was too hot and the temperature change too sudden. Sometimes going back to hot developer then through the other solutions will help unfold such a tissue. Or too weak a gum mastic solution was used.

It is good practice to stain a known positive section along with the specimen for diagnosis as a check on the solutions.

Never use a metal needle for removing tissues. Use the glass rod lifter.

This method is also applicable for paraffin sections, provided period of staining in each step is doubled. It has been used with success by us in demonstrating spirochetes in primary sores where dark-field examinations have been negative. A small piece of tissue of the chancre, after injection of few drops of Novocain solution, is snipped off with pair of sharp scissors, and the above technic carried out.

Krajian's Method of Demonstrating Treponema Pallidum in Smear Preparations

Clean the lesion and scrape off material from ulcerated surface and prepare smears. Flood smears with warm (60° C.) primary solution (p. 2139) for 5 minutes. Dry in air.

Wash in distilled water.

Treat with a weak solution of gum mastic for 2 minutes (3 drops of saturated alcoholic solution of gum mastic mixed with 7 c.c. 95% alcohol).

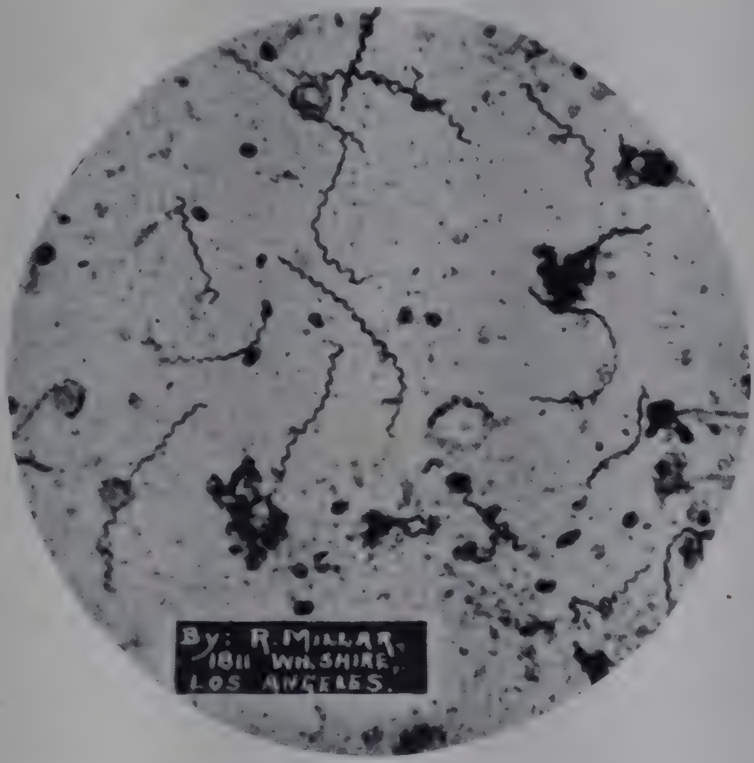

Fig. 627.—*Treponema pallidum* in smear preparations. Krajian's method.

Pour off gum, glow breath over surface of the smear and rinse in distilled water.

Set slide on metal stand or tripod, flood with a 1% silver nitrate solution and heat from under until bubbles arise. (Do not boil.) Keep at that temperature for 3 minutes. Repeat the process once more.

Pour off silver and without washing apply a thin coat of developing solution and leave under electric light for 2 minutes, warming gently with a flame.

The Developing Solution:

Hydroquinone	0.31	gm.
Sodium sulphite	0.06	gm.
Solution of formaldehyde 40% neutral	2.5	c.c.
Pyridin	2.5	c.c.
Saturated solution of gum mastic in 95% alcohol	2.5	c.c.
Distilled water	15.0	c.c.

See page 2139 for directions for making.

Wash with distilled water, dry in filter paper, and examine with oil mimersion lens.

For permanent mounts, dehydrate with absolute alcohol, blot clear in xylol, mount in gum damar.

Staining Mycobacterium Tuberculosis in Sections

Stain for Acid-Fast Bacilli, Method of Fite[1, 2, 3]

(Fuchsin-Formaldehyde Method)

Reagents.—

Fixative.—

Equal parts of 95% alcohol and 10% neutral Formalin.

Carbol Fuchsin.—

New fuchsin (Magenta III)	0.5 gm.
Phenol crystals	5.0 gm.
Methyl alcohol	10.0 c.c.

Distilled water to make 100 c.c. solution.

40% Neutral Reagent Grade Formalin.—

Acid Alcohol.—

See page 2105.

1% Aqueous Potassium Permanganate Solution.—

Dissolve 1 gram of potassium permanganate in enough distilled water to make 100 c.c. of solution.

2% Aqueous Oxalic Acid.—

Dissolve 2 grams of oxalic acid, reagent grade, in enough distilled water to make 100 c.c. of solution.

Harris Hematoxylin.—

See page 2105.

Van Gieson's Mixture.—

Acid fuchsin	0.01 gm.
Picric acid	0.5 gm.

Distilled water to make 100 c.c.

95% Ethyl Alcohol.—

Absolute Alcohol, for paraffin sections.—

98% Isopropanol, for frozen sections.—

Xylol.—

Gum damar.—

See page 2106.

Technic for Frozen Section.—

Fix tissues 24 hours in equal parts of 10% Formalin and 95% alcohol.

Cut frozen sections 10 to 15 microns thick.

Mount in thin celloidin in the usual manner for frozen sections. Page 2104.

Before drying is complete, place in carbol fuchsin for 15 minutes to 48 hours at room temperature, or, better, for 1 hour in the paraffin oven at 58 to 60° C.

Dip in distilled water.

Wash in 40% neutral reagent grade Formalin for 5 minutes. Section turns blue.

Dip briefly in distilled water.

Wash in acid alcohol for 5 minutes. Take care not to lose the section at this point.

Wash by dipping briefly in distilled water.

Wash in 1% aqueous potassium permanganate for 2 to 5 minutes. Section turns brown.

Dip in distilled water.

Wash in 2% aqueous oxalic acid for 40 to 60 seconds or until just a trace of brown remains.

Dip in distilled water.

Stain in Harris hematoxylin for 2 to 5 minutes.

Wash in distilled water.

Place in modified van Gieson's mixture for 3 minutes.

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[1] Fite, G. L.: Am. J. Path. 14: 491, 1938.

[2] Fite, G. L.: J. Lab. & Clin. Med. 25: 743, 1940.

[3] Tilden, I. L., and Tanaka, M.: Am. J. Clin. Path. 15: 95, 1945.

Without washing, place in 95% ethyl alcohol for 2 minutes, then blot the tissue.

Dehydrate in 99% ethyl alcohol or isopropanol. Blot. Absolute alcohol dissolves the celloidin.

Clear in 2 changes of xylol for 5 to 10 minutes each.

Mount in gum damar.

Nuclei stain brown; muscle is yellow; collagenous connective tissue is red; acid-fast bacilli stain deep ultramarine blue; non-acid-fast organisms are unstained.

Technic for Paraffin Sections.—

Fix tissues in equal parts of 95% alcohol and 10% neutral Formalin. Embed.
Cut paraffin sections in the usual manner and mount on the slide.
Stain in carbol fuchsin (above) for 24 to 48 hours at room temperature.
Wash and place in 40% neutral Formalin for 5 minutes.
Wash and place in acid alcohol for 10 minutes.
Wash and place in 1% potassium permanganate for 2 to 5 minutes or until brown.
Wash and place in 2% oxalic acid 20 to 30 seconds.
Wash and stain in Harris hematoxylin for 2 to 3 minutes.
Wash and stain in van Gieson's mixture for 3 minutes.
Do not wash after treatment with van Gieson mixture.
Dehydrate in 95% alcohol for *1 minute only.*
Then place in absolute ethyl alcohol to dehydrate completely.
Clear in several changes of xylol.
Mount in gum damar.
For staining characteristics with this method, see above.

In following this technic be sure that the section turns a deep blue color in Formalin. If this color fails to develop, use a different supply of Formalin. This is the most important step in the procedure.

Staining of Lepra Bacilli in Paraffin Sections

Owing to the lesser degree of acid-fastness of lepra bacilli as compared with tubercle bacilli, Fite, Cambre, and Turner[1] worked out a staining method for demonstration of lepra bacilli in tissues regardless of the technics of fixation and embedding.

Technic.—

Remove paraffin with two changes of the following mixture, allowing a minute or two for each change:

Cottonseed (or peanut or olive) oil	1 part
Xylol	2 parts

Drain, wipe off excess oil and blot to opacity. The residual oil in the section helps to prevent shrinkage and injury of the section.

Remove mercury crystals (if present) with strong solution of iodine, U.S.P. (2 minutes), followed by a hyposulphite or thiosulphate solution rather than alcohol. Wash in tap water.

Stain cold 15 to 30 minutes in any standard preparation of carbol-fuchsin (Ziehl-Neelsen) but not in a concentration solution such as Kinyoun's. Wash.

Decolorize with 1% concentrated hydrochloric acid added to 70% alcohol—not to the point of totality, but leaving a faint pink color. One to two minutes will be required. Wash.

Counterstain with Loeffler's alkaline methylene blue about 30 seconds. Wash in tap water.

Blot, let stand a few minutes to dry, mount directly in a synthetic mounting medium, such as Clarite or Permount.

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[1] Arch. Path. 43: 624, 1947.

Krajian Stain for the Demonstration of Acid-Fast Organisms in Tissue Sections

This method is applicable to both frozen and paraffin sections fixed in ordinary fixatives.

Reagents.—

Carbol Fuchsin.—(See page 1403.)

Krajian Decolorizing Agent.—

Arsenic acid	1 gm.
60% ethyl alcohol	100 c.c.

Loeffler's Methylene Blue.—(See page 1403.)

Anhydrous Isopropanol or Absolute Alcohol.

Iso-Creosote Mixture.—

Equal parts of anhydrous isopropanol or absolute ethyl alcohol and beechwood creosote.

Xylol.

Gum Damar.

Technic.—

Cut frozen sections 7 to 10 microns.

Transfer to a slide, dehydrate, blot in filter paper, dip once in celloidin, and blow over the surface until dry. (See page 2104 for this method of handling frozen sections.) Wash in tap water until the surface is wet.

Place the slide on a tripod or on a warming table, apply carbol-fuchsin, and steam gently with a flame for three minutes.

Discard the staining solution, wash with tap water, and apply arsenic acid alcohol with a medicine dropper two to four times, agitating the slide constantly for even destaining, or until most of the red color is removed.

Wash in tap water. Apply Loeffler's methylene blue solution for two minutes.

Wash in tap water. Dehydrate with three applications of anhydrous isopropanol or absolute ethyl alcohol.

Immediately apply iso-creosote, two or three applications, agitating the slide constantly for even differentiation. This removes the excess blue stain from the tissue as well as from the organisms. This is the crucial step. If overdifferentiated, the background will be very light blue without affecting the organisms; light blue is the right color.

Blot immediately in filter paper.

Clear in three changes of xylol, one minute each.

Mount in gum damar.

For paraffin sections, remove paraffin with two applications of xylol, two minutes each, remove xylol with two applications of isopropanol or absolute ethyl alcohol, wash in tap water, and proceed with method described.

Acid-Fast Tubercle Bacilli Stain*

Gradwohl Modification of Krajian Stain

Cut frozen or paraffin sections. If paraffin sections are used, put through two changes of xylol to remove paraffin.

Place section on slide.

Dehydrate *thoroughly* with isopropanol. Blow and blot.

Dip twice in celloidin. Blow celloidin dry.

Stain lightly with Harris hematoxylin (15 to 20 dips).

Wash in tap water.

Place slide on staining pan and flood with carbol-fuchsin. Steam gently ten minutes.

Pour off carbol-fuchsin and wash thoroughly in tap water.

Decolorize with acid alcohol. *Note:* Make sure that section is thoroughly decolorized—until no further red washes off. Two changes of acid alcohol may be necessary to decolorize completely.

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*This method, which is a modification of Krajian's original method, has been successfully worked out in our laboratories.

Dehydrate with isopropanol.
Dip in 2% aqueous sodium cyanide until background becomes blue (15 to 20 dips).
Wash in tap water.
Dehydrate again.
Clear in two or three changes of xylol.
Mount in gum damar.

Fluorescence Method of Demonstrating Mycobacterium Tuberculosis in Tissues (Tanner)

The use of fluorescence in microscopy for demonstrating *Mycobacterium tuberculosis* in smears has been described on pages 1609 ff. The use of fluorescence microscopy for demonstrating this organism in tissues has been reported in European literature.[1, 2, 3] Tanner[4] gives an excellent method of using this technic for the demonstration of tubercle bacilli in tissue.

Reagents.—

Auramine Stain.—

Solution A.—

Distilled water	87 c.c.
Phenol	3 c.c.

Solution B:—

95% ethyl alcohol	10.0 c.c.
Auramine-O	0.1 gm.

Add solution B to solution A.

Decolorizing Solution.—

70% alcohol	100 c.c.
Hydrochloric acid, c.p., conc.	1 c.c.
Sodium chloride, c.p.	0.5 gm.

0.1% Aqueous Solution of Methylene Blue.—

Dissolve 100 mg. methylene blue in 100 c.c. distilled water, grinding in a mortar, and adding small portions of the solvent until all the dye is in solution. Filter after 24 hours.

Technic.—

Cut sections of tissue blocks, fixed in Formalin and embedded in paraffin, at 5 microns. Mount on clean glass slides with a minimal amount of egg albumin. When the sections are dry, remove the paraffin in the usual manner and cover the deparaffinized sections with the auramine staining solution for five minutes.

Wash the sections and cover with the decolorizing solution for twenty minutes.

At the end of this time, wash the sections with tap water and cover with fresh decolorizer for another twenty minutes.

Wash the tissue with tap water and counterstain with 0.1% aqueous solution of methylene blue for one minute.

Wash. Dry. Examine with the fluorescence microscope without using a coverglass.

The methylene blue gives a black or dark blue background but does not interfere with the fluorescence of the organisms.

Some sections, according to Tanner, gave satisfactory results without the methylene blue, but the use of that counterstain gave a better contrast and it is recommended as a routine procedure in tissue sectioning. The results with both methods are satisfactory, the bacilli standing out as bright yellow rods on a nearly black background. The organism can be identified readily by using an 8 mm. objective and a ×25 ocular.

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[1] Bachman, W., and Finke, L.: Deutsche med. Wchnschr. 65: 1474, 1939.
[2] Schallock, G.: München. med. Wchnschr. 87: 102, 1940.
[3] Finke, L.: Arch. f. Hyg. 123: 381, 1940.
[4] Proc. Staff Meet., Mayo Clinic 16: 839, 1941.

Richards* prefers glycerine mounts, made semipermanent by ringing with gold size. This method permits one to examine adequately about three times as quickly by the fluorescence method as by the carbol-fuchsin method. Tanner found eight more positives by the fluorescence method than by the carbol-fuchsin stain. Ease of preparation and speed of examination are important advantages of the fluorescence method for demonstrating *Mycobacterium tuberculosis* in tissue sections.

Staining Tubercle Bacilli in Tissues (Fuller)

Method No. 1

It is believed by some authorities that fixation of tissues in Formalin renders them unsuitable for staining acid-fast bacteria. Fuller[1] has developed a method which gives good staining of the bacteria with either Formalin or Zenker-formol fixation. With Formalin-fixed tissues the green counterstain is prone to fade in a few months but the bacteria and nuclei remain well stained indefinitely. With tissue fixed in Zenker-formol, all structures retain the dyes for prolonged periods. The methods is as follows:

Solutions.—

Iron Alum, 5% Aqueous.—

Dissolve 5 gm. iron alum in distilled water and dilute to 100 c.c. with distilled water.

Hematoxylin.—

Dissolve 1 gm. certified hematoxylin
in 80 c.c. hot distilled water.
Cool and add 10 c.c. glycerin
and 10 c.c. 95% alcohol.

Picric Acid Solution.—

Add 2 parts of saturated alcoholic solution of picric acid
to 1 part of 95% alcohol.

Fuchsin Solution.—

Add 16 c.c. of saturated alcoholic solution of fuchsin, basic,
to 84 c.c. of aniline water.

Acid Alcohol.—

Add 3 c.c. of nitric acid to enough 95% alcohol to make 100 c.c. of solution.

Alkaline Water.—

Add a few drops of ammonium hydroxide to 100 c.c. distilled water.

Light Green Solution.—

Dissolve 1 gm. light green in enough distilled water to make 100 c.c. of solution.

Technic.—

Run slides down to water as usual and rinse in distilled water.

Mordant in iron alum solution in the paraffin oven for 5 minutes (45° to 50° C.).

Rinse in tap water.

Stain in hematoxylin, also in the paraffin oven, for 5 minutes.

Put directly into the picric acid for 5 minutes, or longer if necessary, until the stain remains only in the nuclei.

Wash thoroughly in running water (15 minutes or longer) until the sections are thoroughly free of the picric acid.

Place on the heating bar and pour the fuchsin solution over them. Heat until steam comes off, but do not boil. Let cool for 3 minutes, heat again, and again allow to cool for 3 minutes.

Destain in acid alcohol for a few seconds, until the fuchsin starts coming off in clouds; rinse in tap water and continue destaining until the sections are barely pink.

Rinse in water and put into alkaline water for a few seconds.

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*Personal communications.

[1] J. Lab. & Clin. Med. 23: 416, 1938.

Wash thoroughly in running water for 10 minutes or more.

Stain in the light green solution for 5 minutes.

Rinse in water and dehydrate quickly through increasing strengths of alcohol and xylol; mount as usual.

This method is excellent for purposes of photomicrography, as tubercle bacilli are stained brilliantly red while nuclei and cytoplasm take different values of green so that excellent contrast is obtainable. For ordinary microscopic examinations, the red-green contrast has been found superior to other staining methods. This method is satisfactory even for tissues such as the brain and meninges which have been very difficult to handle by the usual tubercle bacilli sectional methods of staining.

Method No. 2

Tissue must not have been in Formalin too long.

Overstain in hematoxylin	20 to 30 minutes.
Distilled water	30 minutes.
Carbol-fuchsin	30 to 60 minutes in the incubator at 37° C.
Following solution of hydrochloric acid	1 minute.
Hydrochloric acid	1 part
70% alcohol	100 parts
Wash in 70% alcohol	2 to 3 minutes.

Wash in distilled water.

Dilute lithium carbonate solution until blue.

Concentrated lithium carbonate solution	1 part
Distilled water	10 parts
Distilled water	5 to 10 minutes.
90% alcohol	5 minutes.

Absolute alcohol until dehydrated.
Xylol until clear.
Mount in Canada balsam.

Tubercle bacilli stain red and are characteristic.

Method No. 3

Embed in paraffin or use fixed frozen sections and cut sections 5 microns.
Hydrate through decreasing strengths of alcohol down to water in usual manner.
Place in hot carbol-fuchsin and steam 5 to 10 minutes.
Decolorize in 25% hydrochloric acid.
Wash in distilled water.
Blot.
Place in Loeffler's methylene blue for 1 minute.
Wash in water.
Blot.
Wash in 96% alcohol.
Wash in aniline oil.
Wash in mixture of one-third aniline oil and two-thirds xylol.
Wash in xylol.
Mount in Canada balsam.

Kinyoun Carbol-Fuchsin Method for the Demonstration of Tubercle Bacilli in Tissue

Solution 1.—

Basic fuchsin	2 gm.
Phenol crystals	8 gm.
95% alcohol	20 c.c.
Distilled water	100 c.c.

Solution 2.—

Prepare a 0.5% solution of acetic acid by adding 0.5 c.c. of glacial acetic acid to distilled water and diluting it to 100 c.c. with distilled water.

Dissolve 0.5 gm. methylene blue in this 0.5% acetic acid solution.

Solution 3.—

Add 15 c.c. hydrochloric acid to 95% alcohol and dilute to 100 c.c. with 95% alcohol.

Technic.—

Remove the paraffin from the sections by passing them through three changes of xylol in the usual manner.

Place slides for 1 minute each in the following solutions: absolute alcohol, 95% alcohol, 70% alcohol, distilled water.

Stain with Kinyoun's carbol-fuchsin for 1 hour at room temperature.

Rinse with distilled water.

Decolorize in the hydrochloric acid solution until pink.

Counterstain in the methylene blue solution 1 minute.

Wash in distilled water.

Dehydrate in the usual manner.

Mount in balsam, gum damar, or Clarite.

Methods for the Differential Staining of Gram-Positive and Gram-Negative Bacteria in Tissue Sections

1. Differentiation of Gram-Negative and Gram-Positive Organisms in Frozen Sections (Krajian*)

Krajian has emphasized the importance of a staining method for the demonstration of gram-positive and gram-negative organisms in tissue sections. Ever since Christian Gram[1] in 1884 devised his method for the demonstration of bacteria in tissue sections, various attempts have been made to demonstrate both gram-positive and gram-negative organisms in paraffin sections of tissue fixed in Zenker's solution or in Formalin. Methods have been reported by MacCallum,[2] Lyon,[3] Lillie,[4] Brown and Brenn,[5] Rudnikoff and Stowsky,[6] Glynn,[7] and others. Weigert's[8] modification has been satisfactory for the demonstration of fibrin and bacteria, but it demonstrates only gram-positive organisms.

The methods alluded to have used the original stains of Gram, but no attempt had been made to describe a differential stain for bacteria until Krajian published his method, using a different dye and different chemicals. This method is easy to apply to frozen sections and gives excellent simultaneous staining of both gram-positive and gram-negative organisms. He found that it was successful in staining four different frozen sections mounted on the same slide, one each of a kidney containing colon bacilli, a lung with Friedländer bacilli, a kidney with staphylococci, and a brain containing torulas. The method has been successfully applied to tissues containing streptococci, Welch bacilli, influenza bacilli, monilias, actinomycetes, meningococci, gonococci, and Negri bodies. This method is recommended because it has been found very efficient in our laboratories.

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*This is the modified brilliant green formula. This solution is stable. The original formula for brilliant green solution was 0.3 gram of brilliant green dissolved in 10 c.c. of copper-zinc sulphate solution. This solution remained in good working order for only 24 hours.

[1] Fortschr. d. Med. 2: 185, 1884.

[2] J. A. M. A. 72: 193, 1919.

[3] J. A. M. A. 75: 1017, 1920.

[4] Arch. Path. 5: 828, 1928.

[5] Bull. Johns Hopkins Hosp. 48: 69, 1931.

[6] Arch. Path. 19: 543, 1935.

[7] Arch. Path. 20: 896, 1935.

[8] Ztschr. F. wissensch. Mikr. 4: 512, 1887.

Reagents.—

Harris Hematoxylin.—(Page 2105.)

Acid Alcohol, 1% hydrochloric acid in 70% alcohol (page 2105).

Copper-Zinc Sulphate Solution.—

Copper sulphate	7 gm.
Zinc sulphate	4 gm.
Distilled water	100 c.c.

Dissolve with heat.

Brilliant Green Solution.*—

Hematoxylin crystals	0.3 gm.
Commercial Formalin (40%)	10.0 c.c.

Dissolve and add

Brilliant green	0.3 gm.

Mix thoroughly. If a precipitate forms, filter.

This solution keeps indefinitely.

Ammonium Nitrate, 5% Solution.—

Dissolve 5 gm. ammonium nitrate in 100 c.c. of distilled water.

Carbol Fuchsin.—(See page 1403.)

Dioxane.—This may be obtained from the Carbide and Carbon Chemicals Corporation, 30 E. 42 St., New York 17, N. Y.

Creosote-Xylol Mixture.—

Mix equal parts of beechwood creosote and chemically pure xylol, using dry glassware and dry container. Take care that no water enters the mixture.

Technic.—

Prepare frozen sections in the usual manner (pages 2102 ff.).

Stain in Harris hematoxylin for two minutes. It is easy to carry out this procedure in Stender dishes, using a glass lifter to move the sections from one solution to the other.

Wash in tap water until blue and decolorize rapidly in acid alcohol, dipping in and out five to seven times.

Rinse in tap water.

Place in copper-zinc sulphate solution for 3 minutes.

Stain in brilliant green solution for 5 minutes.

Rinse in water.

Fortify with 5% aqueous ammonium nitrate for one minute.

Rinse in tap water, and mount on a glass slide.

Stain in carbol-fuchsin for 2 minutes.

Pour off, and without washing, apply creosote-xylol mixture, changing the mixture several times and agitating the slide for even differentiation until the background appears to be clear red with no more stain leaving the section. This step requires about one minute. It should be controlled under the microscope.

Clear in pure xylol for two minutes.

Mount in gum damar.

Nuclei are bluish red, gram-positive organisms bluish green, gram-negative organisms red, monilias and actinomycetes green, and Negri bodies bright red with greenish chromatin bodies.

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*See footnote * p. 2148.

2. Gram Stain of Tissues and Smears

(Second Method of Krajian)

This method is applicable to both sections and smears. It is recommended.

Reagents.—

Loeffler's Methylene Blue.—(See page 1403.)

Isopropanol, Anhydrous.

Creosote-Xylol Mixture.—

Creosote	1 part
Xylol	3 parts

Creosote-Fuchsin.—

Creosote	1 part
Xylol	3 parts

Use 50 c.c. of this mixture to 3 c.c. of 6% alcoholic solution of basic fuchsin.

Gum Damar.

Technic.—

Stain section or smear (fixed in the usual manner by heat) with Loeffler's methylene blue for 2 minutes.

Wash, and dehydrate with isopropanol, two applications.

Dip in creosote-xylol for 10 seconds or until most of the color has been removed.

Apply creosote-fuchsin for 10 to 15 seconds, agitating the slide until background is deep red.

Pour off.

Apply creosote-xylol until section or smear is clear.

Xylol for further clarification.

Mount in gum damar. (The last two steps are unnecessary when dealing with smears.)

3. Gram-Weigert Method (Weigert)

Fix in Zenker's fluid.

Embed in paraffin or celloidin.

Cut sections and stain as follows:

Reagents.—

Alum Hematoxylin.—

Dissolve 1 gm. hematoxylin crystals in 100 c.c. distilled water by the aid of gentle heat.

Dissolve 20 gm. ammonium or potassium alum in 300 c.c. distilled water.

Add the solution of hematoxylin to the alum solution and mix.

Add 1 gm. thymol to prevent growth of molds.

Ripen for ten days in a flask or bottle lightly stoppered with a plug of cotton.

Keep in a tightly stoppered bottle.

2.5% Aqueous Solution of Eosin.—

Dissolve 2.5 gm. eosin Y in 100 c.c. distilled water.

Aniline Crystal Violet, Sterling.—

Crystal violet, 85% dye content	5.0 gm.
Aniline	2.0 c.c.
Alcohol	10.0 c.c.
Distilled water	88.0 c.c.

Mix the aniline with the alcohol and dissolve the dye in the mixture. Then add the distilled water, mix well, let stand 24 hours or more, and filter.

Gram's Iodine Solution.—(See under Method 4.)

Aniline, c.p.

Xylol.

Canada Balsam.

Technic.—

Stain sections lightly in alum hematoxylin.
Wash in several changes of tap water.
Put sections in 2.5% aqueous eosin for 5 to 15 minutes in the paraffin oven.
Stain in Sterling's crystal violet for ½ hour.
Wash in water.
Treat with Gram's iodine solution 1 to 2 minutes.
Wash in water.
Blot with filter paper and decolorize and clear in several changes of aniline.
Rinse in xylol and mount in Canada balsam.

With this stain, gram-positive organisms are deep violet, nuclei blue to violet, connective tissue red, gram-negative bacteria unstained.

4. Krajian's Modification of the Gram-Weigert Method

Reagents.—

1% Aqueous Eosin Solution.—

Dissolve 1 gm. of eosin Y in enough distilled water to make 100 c.c. solution.
Filter after 24 hours.

Sterling's Gentian Violet.—

Gentian violet	5 gm.
95% alcohol	10 c.c.
Aniline oil	2 c.c.
Distilled water	88 c.c.

Dissolve the aniline oil in the distilled water by shaking violently until solution is effected. Then filter through filter paper. Dissolve the gentian violet in the alcohol. Now add the aniline-oil water to the dissolved gentian violet. Shake well, but do not filter. The solution will keep for several weeks to several months.

Gram's Iodine Solution.—

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	300 c.c.

Mix the iodine and the potassium iodide thoroughly in mortar. Add the water a small amount at a time until all the iodine is in solution. Filter after 24 hours.

Aniline Oil-Xylol.—

Mix equal parts of aniline oil and xylol in a clean dry container.

Technic.—

Cut frozen sections as directed, and mount on a slide under celloidin (page 2105).
Stain in 1% aqueous solution of eosin for 5 minutes.
Wash in water.
Stain in Sterling's gentian violet for 3 minutes.
Wash with Gram's iodine solution, then stain in Gram's iodine solution for 3 minutes.
Blot with several thicknesses of Whatman No. 1 filter paper.
Decolorize several times in aniline-oil-xylol mixture.
Clear in 2 changes of xylol.
Clean excess celloidin from around section and mount in gum damar.
Gram-positive organisms and fungi stain violet.

5. Brown and Brenn[9] Gram Staining of Tissues

Reagents.—

Harris Hematoxylin.—(Page 2105.)

3% Acid Alcohol.—

Add 3 c.c. hydrochloric acid, conc., c.p., to enough 95% alcohol to make 100 c.c. solution.

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[9] Bull. Johns Hopkins Hosp. 48: 69, 1931.

Ammonia Water.—

Add 1 c.c. ammonium hydroxide, c.p., to 100 c.c. distilled water.

5% Aqueous Solution of Sodium Bicarbonate With Phenol.—

Dissolve 5 gm. sodium bicarbonate, c.p., anhydrous, in 100 c.c. of 0.5% phenol.

1% Aqueous Gentian Violet.—

Dissolve 1 gm. gentian violet crystals in 100 c.c. distilled water.

Lugol's Solution (Page 1401).

Decolorizer.—

Add 1 part of ether to 3 parts of acetone.

Rosanilin Hydrochloride.—

Dissolve 0.005 gm. rosanilin hydrochloride in 100 c.c. distilled water.

Dilute Basic Fuchsin Solution.—

Add 0.1 c.c. saturated alcoholic solution of basic fuchsin to 100 c.c. distilled water.

Acetone.

Picric Acid Differentiater.—

Dissolve 0.1 gm. of picric acid in 100 c.c. acetone.

Acetone-Xylol Mixture.—

Equal parts of acetone and xylol, made in dry containers.

Xylol.

Balsam.

Technic.—

Prepare paraffin sections.

Stain in freshly filtered Harris hematoxylin for 2 to 5 minutes.

Wash in acid alcohol until light pink.

Wash in ammonia water until blue.

Wash in water.

Mix, in a small vial, 5 drops of the sodium bicarbonate-phenol solution with about 0.75 c.c. of 1% aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for two minutes.

Wash quickly with water.

Cover with Lugol's solution for one minute.

Wash with water. Blot.

Decolorize in ether-acetone, dropping it onto the slide until no more color comes off. Blot.

Stain for 5 minutes with rosanilin hydrochloride or basic fuchsin.

Wash in water. Blot but do not allow the section to dry.

Pass through acetone.

Decolorize and differentiate by dropping over the section the 0.1% picric acid in acetone until the section becomes a yellowish pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the fuchsin should be decolorized from the tissue, but the gram-negative bacteria should remain red.

Pass successively through acetone, equal parts of acetone and xylol, and xylol.

After clearing in xylol mount in balsam.

(Beginning with the fifth step it is best to work with only one slide at a time.)

Cell nuclei should be stained dark reddish brown; cytoplasm, yellowish; gram-positive bacteria, deep violet or almost black; gram-negative bacteria, bright red.

Leukocytes generally stand out plainly with a dusky, yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be yellow or red, depending upon the degree of decolorization in picric acid. Cartilage stains pink. Striated muscle and fibrin generally stain yellow but may retain more or less of the red stain. The tissue stain has many of the characteristics of van Gieson's stain.

By this method it is possible to stain successfully sections of tissue showing influenza bacilli, pneumococci, and streptococci; pertussis bacilli crowded among the cilia of the epithelial cells of the trachea and bronchi; gonococci, staphylococci, and streptococci in heart valve vegetations; meningococci in the meninges; *Corynebacterium pyogenes, Actinomyces necrophorus, Bacillus arthracis, Clostridium perfringens,* and *Clostridium septicum* in animal tissues.

6. MacCallum's Modification of Goodpasture's Method

Fix in Zenker's solution and embed in paraffin.

Goodpasture stain used in this technic is as follows:

Basic fuchsin	0.59	gm.
Aniline	1.0	c.c.
Phenol crystals	1.0	gm.
Alcohol, 30 per cent	100	c.c.

Technic.—

Stain sections for 10 to 30 minutes in Goodpasture's stain.

Wash in water.

Differentiate in strong Formalin for a few seconds, until the bright red color changes to a clear rose.

Wash in water.

Counterstain in a saturated aqueous picric acid solution (about 1.22 per cent) 3 to 5 minutes or less, until section assumes a purplish yellow color.

Wash in water.

Differentiate in 95 per cent alcohol. This causes the red color to reappear; some of it is washed out together with some of the yellow color.

Wash in water.

Stain for 5 minutes in Sterling's crystal violet solution. (Page 2151.)

Wash in water.

Immerse in Gram's iodine solution for 1 minute.

Blot dry without washing in water.

Treat with equal parts of aniline and xylol until no more color comes away.

Rinse with 2 changes of xylol and mount in balsam.

With this method, gram-positive organisms are blue; gram-negative organisms, red; all other tissue elements, various shades of red to purple.

Staining Iron Pigments in Frozen Sections (Krajian)[1]

Reagents.—

Potassium Ferrocyanide in Hydrochloric Acid.—

Freshly prepared 2% aqueous solution of potassium ferrocyanide	1 part
Hydrochloric acid, 1%	3 parts

Heat the mixture in a clean Pyrex test tube until beads of vapor form on the inner surface of the glass (about 70° C.). Use this mixture while hot.

Eosin, 1% Aqueous.—(1 gm. eosin Y in 100 c.c. distilled water.)

Technic.—

Prepare frozen sections in the usual manner. (See page 2104.)

Place single sections in hot potassium ferrocyanide in hydrochloric acid either in a Pyrex test tube or in a small Stender dish and leave in the hot solution for ½ to 2 minutes.

Wash thoroughly in several changes of distilled water.

Now mount on a glass slide, dehydrate, and dip in celloidin as directed on page 2105.

Counterstain to the correct color with 1% aqueous eosin.

Rinse in distilled water.

Dehydrate through two changes of absolute alcohol.

Clear in pure xylol.

Mount in gum damar.

Nuclei and hemofuscin stain bright red; hemosiderin stains blue.

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[1] Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, The C. V. Mosby., 1952.

Staining of Iron or Hemosiderin in Paraffin or Frozen Sections

Make paraffin or fixed frozen section as usual.

Dehydrate through xylol and decreasing strengths of alcohol down to water as usual.

Stain in warm potassium ferrocyanide 2% fresh solution (doubly distilled water) for 3 minutes until a blue color appears. Heat in beaker but not enough to turn color.

Wash in several changes of doubly distilled water.

Stain 10 to 30 minutes in fuchsin:

Fuchsin	0.5 gm.
Alcohol (95%)	50.0 c.c.
Water	50.0 c.c.

Or 0.5% basic fuchsin in 50% alcohol for 10 to 20 minutes.

Differentiate and dehydrate in 95% alcohol.

Wash in absolute alcohol.

Wash in xylol.

Mount with balsam.

Hemosiderin or iron is blue or blue-green.

Weigert's Myelin Stain

Use Formalin-fixed tissue embedded in celloidin. After cutting:

Wash in water.

Place in the following mordant for 12 to 24 hours:

Potassium bichromate	2.0 gm.
Flurochrome	2.5 gm.
Water	100 c.c.

Wash in water 2 to 3 hours (few minutes to 1 to 12 hours).

Wash in 70% alcohol for 10 to 15 minutes.

Wash in water 10 to 15 minutes to 12 hours.

Immerse in copper acetate one-half saturated solution 1 hour (filter) until blue.

Wash in water a few minutes to remove copper.

Stain two hours:

Hematoxylin	0.75 gm.
Alcohol (96%)	10.00 c.c.
Water	90.00 c.c.

Make fresh each time.

Decolorize in:

Borax	2.0 gm.
Potassium ferricyanate	2.5 gm.
Water	200.0 c.c.

Dilute the decolorizer 1:4 or 1:10 if the tissue is to be left overnight.

Wash thoroughly in running water.

Dehydrate in 80, 96, and 99% alcohols.

Wash in carbol-xylol and xylol.

Mount in balsam.

The Oxydase Staining of Tissues

Method of Winkler, W. Schultze

Reagents.—

10% Formalin.—(4% formaldehyde)—Dilute 10 c.c. Formalin (40% formaldehyde) to 100 c.c. with tap water.

1% Aqueous Alpha-Naphthol.—Place 1 gram of alpha-naphthol in 100 c.c. of 1% potassium hydroxide. Heat. Only a part dissolves. Filter.

Diluted Lugol's Solution.—Dilute Lugol's solution with an equal volume of distilled water and filter.

Lithium Carbonate Water.—Use a saturated solution.

1% Dimethyl-para-phenylenediamine.—Hermetically sealed ampules containing 0.5 gm. each may be purchased. Solution does not keep longer than a few days.

Dissolve the contents of one ampule in 50 c.c. of either distilled water or physiologic saline.

Allow to stand one hour, shaking frequently.

Filter.

Weak alum hematoxylin, such as Delafield's hematoxylin diluted, may be used.

Technic.—

(A) **Rapid Method—Not Permanent.**—Fix the tissue in the 4% formaldehyde (10% Formalin).

Freeze and cut.

Place in 1% alpha-naphthol for several minutes, depending on thickness.

Place in 1% dimethyl-para-phenylenediamine solution for a short time, until the leukocytes turn blue. When the leukocytes are blue, rinse, and examine in water or glycerine under a cover glass.

(B) **Permanent Stain.**—Carry out the above technic.

Pour the rinsed preparations into dilute Lugol solution for 3 minutes.

Flood with a large volume of lithium carbonate water for 24 hours.

Counterstain with weak hematoxylin.

Mallory Phosphotungstic Acid-Hematoxylin Stain

Zenker fixation is preferred for general use, although Formalin- or alcohol-fixed tissues can be used for some purposes.

Staining Solution.—

Hematoxylin	1 gm.
Phosphotungstic acid	20 gm.
Distilled water	1000 c.c.

Dissolve the ingredients in separate portions of the water.

Dissolve the hematoxylin with gentle heat.

Cool and mix. No preservative is necessary. Allow to ripen for several weeks, or ripen at once by adding 0.177 gm. of potassium permanganate or 10 c.c. of a freshly prepared 0.25% aqueous solution of potassium permanganate for every 100 c.c.

Technic.—

If Zenker-fixed tissues are used, treat cut sections in the usual manner with iodine to remove the mercury precipitate.

Extract the iodine with alcohol or with a 0.5 per cent solution of sodium thiosulphate for several minutes.

Rinse thoroughly in water.

Place sections in 0.25 per cent aqueous solution of potassium permanganate for 5 to 10 minutes.

Wash in water.

Place in 5 per cent aqueous solution of oxalic acid for 10 to 20 minutes.

Wash thoroughly in several changes of water.

Stain in phosphotungstic acid-hematoxylin for 12 to 24 hours.

Place directly in 95 per cent alcohol, then transfer to absolute alcohol to dehydrate. Dehydrate quickly because alcohol readily extracts the red part of the stain.

Clear in xylol and mount in balsam or gum damar.

If celloidin sections are used, clear with xylol after the 95 per cent alcohol, by means of filter paper and blotting. Origanum and other oils cause the blue stain to fade.

Nuclei, centrioles, achromatic spindles, and fibroglia, myoglia and neuroglia fibrils, fibrin and the contractile elements of striated muscle are stained blue; collagen (including reticulum) and the ground substances of cartilage and bone stain varying shades of yellowish to brownish red. Coarse elastic fibrils are sometimes colored a purplish tint.

Peritoneal or Pleural Fluid, etc.

See **Puncture Fluids**, Chapter VII, page 1222 for other methods of preparation.

The serumizing technic of Krajian (page 2107) is recommended for the sediment. Another method is as follows:

1. Centrifuge material to obtain the sediment.
2. Accumulate enough fairly solid sediment to work with.

3a. Fix in Formalin, etc., as above, and proceed

or

3b. Make a smear of the sediment and stain as a paraffin section after preliminary heating in the flame. Mount after staining.

Blood and Uterine Scrapings

Use the Krajian Serumizing Method, page 2107.

In sectioning the above, do not allow xylol and paraffin mixture to become warmer than merely the melting point, otherwise the blood will crack.

Decalcification of Bone for Paraffin Sections

Place a specimen in 5% nitric acid and change daily until the specimen becomes soft. This requires from one to five days. Be sure to use a very large quantity of solution.

Wash in running water twenty-four hours and then carry through the alcohols from 80 to 95 per cent to absolute.

Place in cedarwood oil for clearing until it becomes transparent and sinks.

Place in xylol for thirty minutes.

Place in paraffin in oven for two hours.

Embed in paraffin and cut sections as usual.

Stain preferably with eosin-methylene blue.

Instead of using 5 per cent nitric acid and washing in running water for twenty-four hours, the following decalcifying solution may be used, taking care to use 100 times volume of tissue:

Hydrochloric acid	4 c.c.
Glacial acetic acid	3 c.c.
Chloroform	10 c.c.
Water	10 c.c.
Alcohol (95%)	73 c.c.

Pass tissue immediately into fresh absolute alcohol (two changes), then into cedarwood oil; the rest of the technic is as above.

A rapid method of decalcification is to place the tissue in the following solution:

Formalin	1 part
Nitric acid	1 part
Water	9 parts

Wash in running water and then begin dehydration in 80% alcohol and continue through the alcohols a little more slowly.

Bone Tissue (or Calcified Nodules)

Decalcify in 5% nitric acid or

10 c.c. formaldehyde (40%)
90 c.c. distilled water
20 c.c. nitric acid.

If the bone is not too large, keep in for two or three hours. It may be kept in the solution as long as forty-eight hours without harming it. There should be at least ten times as much solution as tissue. Change the solution frequently.

Wash in running water twelve to twenty-four hours.

80% alcohol.

Proceed as for paraffin embedding, pages 2120 ff.

If tissue is soft, it is ready for process.

Bone Marrow Puncture

Bone marrow puncture is discussed on pages 914 ff.

Amyloid Stain

Amyloid Stain of Frozen Sections (Krajian)[1]

Reagents.—

Harris Hematoxylin.—(See page 2105.)

Congo Red Solution.—

Congo red, 4% aqueous solution	9 c.c.
Glycerin, redistilled	3 c.c.

Technic.—

Cut frozen sections in the usual manner. (See page 2103.)
Stain unmounted sections in Harris hematoxylin in a small Stender dish for 1 minute.
Wash in tap water until blue.
Stain in a small Stender dish of the Congo red solution for 10 minutes.
Wash rapidly but thoroughly.
Transfer the section to a slide as directed on page 2104.
Blot with several thicknesses of Whatman No. 1 filter paper.
Dehydrate rapidly, applying isopropanol from a drop bottle several times.
Dip in thin celloidin (page 2105).
Blow breath over section to dry.
Dehydrate twice again with anhydrous isopropanol from a drop bottle.
Clear in a staining jar of xylol for 2 minutes.
Remove excess celloidin from around the tissue, and mount in gum damar.
Amyloid is rose color; nuclei are blue.

Amyloid Stain for Paraffin Sections, Method of Lieb

Crystal Violet Solution.—

Saturated alcoholic solution of crystal violet	10 c.c.
Hydrochloric acid	1 c.c.
Distilled water	300 c.c.

Abopon.—

Mix 100 gm. Abopon with 50 c.c. distilled water.

Technic.—

Deparaffinize the section as usual.
Stain for 2 minutes with crystal violet solution.
Rinse in distilled water.
Mount in Abopon.
Amyloid stains red.

Stains for Fat

The staining of fat necessitates the use of fixatives which will not dissolve fat globules. Fat globules are soluble in ether, chloroform, alcohol, xylol, benzine, etc. Formalin fixes the whole fat-containing material without dissolving it.

Sudan III Method

Place frozen sections in 50% alcohol for a few minutes.

Stain in a saturated solution of Sudan III in hot 70% alcohol which should be filtered before use for 5 to 10 minutes.

One minute in 50% alcohol.

Counterstain in Delafield's hematoxylin 3 to 5 minutes.

Differentiate in 3 to 5% hydrochloric acid, followed by distilled water to which has been added a few drops of ammonia.

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[1] Krajian and Gradwohl: Histological Technic, The C. V. Mosby Co., 1952.

Wash in water 10 minutes.
Mount in gelatin or glycerin gelatin.
Fat stains yellow or yellowish red.

Scharlach R Fat Stain, Frozen Sections (Krajian).[1] Method No. 1

Reagents.—

70% Alcohol.—

70 c.c. of 95% alcohol diluted with distilled water to make a total of 95 c.c.

Scharlach Red.—

Scharlach Red	5 gm.
70% alcohol	50 c.c.
Acetone	50 c.c.
Benzoic acid	1 gm.

The benzoic acid content prevents deterioration of the staining solution and serves to deepen the staining qualities.

Harris Hematoxylin.—(See page 2105.)

2% Sodium Cyanide.—

2 gm. of sodium cyanide dissolved in 100 c.c. distilled water. *POISON.*

Glycerin Jelly (Kaiser).—

French sheet gelatin	10 gm.
Distilled water	60 c.c.

Dissolve the gelatin in the water with heat. Then add a mixture of

Pure glycerin	70 c.c.
Carbolic acid	1 c.c.

Stir the mixture until smooth. Filter through cotton moistened with hot water, carrying out the filtration while the solution is still hot. Store in a cork-stoppered bottle. To melt, place the bottle in the paraffin oven for 15 minutes.

Technic.—

Fix tissues in Formalin, and cut frozen sections in the usual manner (page 2103).
Place sections in a bowl of tap water.
Pick up sections with glass lifter and pass through 70% alcohol quickly.
Stain in a Stender dish containing the Scharlach R solution for 3 to 5 minutes.
Pick sections out of the stain with the glass lifter and pass quickly through 70% alcohol.
Rinse in tap water.
Stain in Harris hematoxylin for thirty seconds.
Lift out with glass lifter and wash with water.
Differentiate with 2% aqueous sodium cyanide for ten seconds.

Wash in water. Place on glass slide. Stand slide against dish to drain off the excess water. Do not allow it to become too dry; a slightly moist surface is desirable.

Mount in warm glycerin jelly.
Fat globules are brilliant red. Cholesterins are less intensely stained.
Nuclei are dark blue. This is a rapid and permanent stain.

Scharlach R Stain for Fat.* Method No. 2

The most commonly used stain for fat is Scharlach R (scarlet red). Sudan III was used originally, but it is less brilliant.

Fix tissues in 10% formaldehyde and cut frozen sections in usual manner.

Pass the sections quickly through 70% alcohol.

Place them in the following staining solution from 3 to 5 minutes: Scharlach R (saturated) in equal parts of 70% alcohol and pure acetone.

Wash quickly in 70% alcohol.

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[1] Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, The C. V. Mosby Co., 1952.

*Quoted from Krajian, A. A.: Histological Technic, 1940, page 114.

Rinse in water.

Counterstain in Harris' hematoxylin for 15 to 25 seconds.

Wash in water until blue.

Differentiate in 2% potassium iodide solution (aqueous).

Wash in water, transfer to slide and drain off water.

Mount in warm glycerin jelly.

True fats stain intensely, cholesterins less intensely.

This is a rapid and permanent stain. It is highly recommended.

Osmic Acid Method

Flemming Solution.—

A. 1% aqueous chromic acid 11 parts, glacial acetic acid 1 part, distilled water 4 parts.

B. Osmic acid 2% in 1% chromic acid solution.

Just before using, mix four parts of (A) with one part of (B).

Use ten times the volume of the object.

Fix in Flemming's solution 2 to 72 hours.

Wash in water 24 hours.

Make paraffin sections.

Make contrast stain with a saturated alcoholic safranin (saturated in absolute alcohol and diluted with equal parts of distilled water before use) 24 hours.

Wash in water.

Acid alcohol (3 drops of concentrated hydrochloric acid to 100 c.c. of alcohol).

Absolute alcohol until no more color comes off.

Xylol.

Balsam.

Nuclear structures are red and fat is black.

Osmic Acid Method for Frozen Sections (Krajian)

Fix in 10% Formalin for 24 hours or longer.

Cut frozen sections.

Place sections in a 1% aqueous solution of osmic acid for 24 hours. (Flemming's or Marchi's solution may be used instead.)

Wash under running water for 6 to 12 hours.

Place in absolute alcohol for several hours to obtain the secondary staining of fat not yet colored.

Oil Red O Method for Fat in Paraffin Sections

Reagent.—

Oil red O	1 gm.
Acetone	50 c.c.
70% alcohol	50 c.c.

Technic.—

Stain for 1 minute in oil red O solution.

Wash in distilled water.

Counterstain in Harris hematoxylin for 1 to 2 minutes.

Develop the blue of the hematoxylin in tap water.

Blot as dry as possible.

Clear in aniline.

Mount in glycerol.

Fat is stained red.

Cajal's Sublimate-Gold Method for Neuroglia[1]

Harden fresh pieces of human cerebrum for 2 to 6 days in:

Ammonium bromide	2 gm.
Formol	14 c.c.
Distilled water	100 c.c.

Section by freezing microtome.

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[1] Ramon-Cajal: Histology, English Translation, Fernan-Nunez, Williams and Wilkins Co., 1933, p. 685.

Wash rapidly in distilled water, and place in:

Gold chloride, 1% aqueous solution	10 c.c.
Mercuric chloride (needles)	8-10 gm.
Distilled water	40-50 c.c.

Allow tissues to remain (in darkness or subdued light) from four to ten hours, or until they acquire an intense purple tone. A temperature of 18° to 22° C. favors the reaction. When the method is used in tissues difficult to stain, such as the lower vertebrates, the temperature is elevated to 25° or 30°. (Castro).

Wash in distilled water, and fix in:

Sodium hyposulphite (saturated solution)	10 c.c.
Sodium bisulphite (normal solution)	2-3 drops

Wash the sections in 50% alcohol. Dry on the slide with fine, clean filter paper. Dehydrate in absolute alcohol, oleum origani cretici, xylol, balsam.

If the reaction turns out well, the cells of neuroglia are stained purple, with the neurones and vessels a light violet. The nerve fibers are little or not at all visible.

This procedure gives especially good results with the adult human cerebrum. However, acceptable preparations are obtainable in the cat and dog, but not very good ones in the rabbit. Fresh tissues are essential.

Method for Staining Nerve Protoplasm*

Nissl's Method for Chromatic Granules

Harden in 95% alcohol.

Section without embedding. To secure the tissue to the cork block, for cutting, use a concentrated solution of gum arabic, which will harden in the alcohol.

Cover sections with the following stain and warm until steam is given off:

Methylene blue	3.75 gm.
Venetian soap (or castile soap)	1.75 gm.
Water	1,000.0 c.c.

A 0.5% aqueous solution of toluidin blue may replace the methylene blue.

Dry the sections with filter paper on the slide and differentiate in:

Aniline oil	10 c.c.
Alcohol 95%	90 c.c.

Dry again, clear in cajuput oil, and then in benzene.

Mount in resin damar dissolved in xylol.

The chromatic granules and the nucleoli stain an intense blue.

In Nissl's method the unembedded sections are difficult to handle. The following modification is preferable:

Harden small pieces of fresh nerve centers 4 to 8 days in 95% alcohol.

Dehydrate, and embed in celloidin.

Stain very fine sections for 10 minutes or longer, in a saturated or very concentrated solution of toluidin blue (Nissl) or thionin (Lenhossek).

Wash in 95% alcohol until of a light blue shade. A little aniline oil may be added to the alcohol to accelerate the decolorization. The background may be stained with erythrosin for contrast.

Clear in xylol and mount in xylol-balsam or gum damar.

Method for Demonstration of Neurones[1]

Cajal's Modification of Golgi's Rapid Method

Harden very small pieces for 1 to 3 days in

Potassium bichromate, 3%	20 c.c.
Osmic acid, 1%	6 c.c.

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*Ramon-Cajal: Histology, English Translation, Fernan-Nunez, Williams and Wilkins Co., 1933, p. 680.

[1] Ramon-Cajal: Histology, William Wood & Company, 1933, p. 681.

Wash for 2 to 4 seconds in distilled water.

Immerse for 30 hours in a 20% solution of crystallized silver nitrate.

Harden for 30 minutes in 95% or absolute alcohol.

Mount superficially in a paraffin block, or between 2 pieces of willow pith for sectioning with the microtome. Make thin sections.

Collect the washed sections in 95% alcohol or absolute alcohol, which is changed 6 or 8 times during the 30-minute period.

Clear for 2 to 5 minutes in oil of cloves.

Transfer rapidly to the slides, and irrigate for same seconds with xylol to remove the oil of cloves.

Apply resin damar dissolved in xylol to the sections.

The sections are dried in the open, so that the varnish may harden in the depths of the tissue and the cells appear as if incrusted in glass. Slow hardening of the varnish, or mounting in the ordinary way, ruins the staining. In order to use the immersion lens, it is advisable to mount the preparations on a coverslip over a perforated piece of wood, with the sections underneath.

Myelin Sheath Stain, Krajian*

Fix tissues in 10% Formalin, cut frozen sections 10 to 15 microns.

Place sections in a 4% Congo red in glycerin, 10 minutes (Congo red 4%, 8 c.c.; glycerin 2 c.c.).

Wash thoroughly in tap water.

Place in a 2% aqueous solution of potassium iodide for 2 minutes.

Wash and place in a 3% aqueous solution of potassium cyanide for 2 minutes.

Wash thoroughly in tap water.

Place in a 10% aqueous ferric ammonium sulphate for 10 minutes at 55° C.

Wash in tap water.

Place in a 20% ripe hematoxylin solution for 5 minutes (2 c.c. ripe hematoxylin, 8 c.c. distilled water).

Wash in tap water.

Destain in a 2% aqueous ferric ammonium sulphate solution until section appears light blue. This takes place in 30 to 45 seconds.

Wash thoroughly in tap water.

Differentiate in a 0.15% aqueous potassium permanganate 10 to 20 seconds.

Wash in tap water.

Place in a 3% aqueous sodium cyanide solution until sections flatten out and assume a light blue appearance.

Wash in tap water, attach to the glass slide.

Dehydrate in 2 changes of absolute alcohol.

Oil origanum 2 minutes.

Pure xylol 2 to 5 minutes.

Mount in gum damar.

The above method has all the advantages of the longer methods, showing very fine details of the fibrils, and yet it can be completed in one hour. It is very highly recommended.

Demonstration of Urea Crystals in Brain Tissue

The following technic, originated by Krajian,[1] is very useful in differentiating at autopsy actual cases of uremia from nonuremic cases. In other words, it has been found that cases with urea crystals in brain tissue are followed by uremic death, while cases that have simulated it do not show urea crystals in the brain tissue.

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*Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, The C. V. Mosby Co., 1952.

[1] Krajian, A. A.: Histological Technique, Handbook, Published by A. A. Krajian, Los Angeles.

Fix very thin brain tissue, about 2 mm. in thickness, in xanthydrol (5 gm. of xanthydrol is dissolved in 100 c.c. of pure glacial acetic acid) for 6 hours.

Wash under running water for one hour.

Fix in 10% Formalin 6 hours or longer. (In urgent cases, a rapid fixation with heat could be resorted to.)

Wash in tap water.

Cut frozen sections 5 to 10 microns.

Transfer to glass slide.

Pour several drops of absolute alcohol over section from a drop bottle, and blot. Repeat this process once more.

Cover with thin celloidin, dipping slide in a wide mouth bottle containing the celloidin.

Fix to slide by blowing the breath over section.

Stain in 1% aqueous eosin solution for several minutes. (1 gm. eosin in 100 c.c. distilled water.)

Wash in tap water.

Dehydrate in three changes of absolute alcohol (drop bottle method).

Place in carbol-xylol for 2 minutes.

Clear in two changes of pure xylol, 1 minute in each.

Mount in gum damar.

Fixation of very thin blocks in xanthydrol solution is very important on account of its poor penetrating power. Staining nuclei with hematoxylin has caused the disappearance of urea crystals; for this reason, staining the background with eosin will be satisfactory.

Van Gieson Stain

10% Hematoxylin.—

Hematoxylin	10 gm.
Absolute alcohol	90 c.c.

Ferric Chloride-Hydrochloric Acid Mixture.—

Ferric chloride ($FeCl_2 \cdot 6H_2O$), 48.3% aqueous solution	4 c.c.
Distilled water	95 c.c.
Hydrochloric acid	1 c.c.

Hematoxylin for Use (Iron-Hematoxylin).—

Mix equal parts of the 1% hematoxylin and iron chloride solutions.

Van Gieson Mixture.—

Concentrated aqueous acid fuchsin	3 c.c.
Saturated aqueous solution of picric acid	150 c.c.

Test this stain for color tone by placing a drop on a piece of filter paper. The proper color tone is seen when there is a yellow center with a lighter peripheral ring.

Technic.—

Stain 3 to 5 minutes in iron-hematoxylin mixture.

Wash in distilled water.

Stain in van Gieson mixture 2 to 3 minutes.

Wash in distilled water.

Dehydrate through increasing strengths of alcohol.

Clear in xylol and mount in gum damar or balsam.

This stain shows the nuclei of the connective tissue as dark greenish; the muscle fibers are light red; and amyloid tissue is brownish yellow.

Van Gieson Stain of Frozen Sections, Krajian Modification[1]

Reagents.—

Elastic Tissue Stain (see page 2163).

Acid Alcohol (see below).

Van Gieson Stain (see page 2163).

Oil Origanum.—Equal parts of oil origanum and absolute alcohol or isopropanol.

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[1] Krajian, A. A. and Gradwohl: Histopathological Technic, The C. V. Mosby Co., 1952.

Technic.—

Cut frozen sections in the usual manner and mount under celloidin. See pages 2102 to 2105 for details.

Stain in the elastic tissue stain for 5 minutes.

Wash in water.

Decolorize in acid alcohol until no more color comes off.

Wash in water for a few seconds.

Dehydrate with isopropanol from a drop bottle and recelloidin the slide. This step prevents the tissue from coming off the slide.

Wash in water.

Stain for 5 to 10 minutes with van Gieson stain.

Wash in water.

Blot dry with several thicknesses of Whatman No. 1 filter paper.

Place in oil origanum for 5 minutes.

Clear in xylol. Remove excess celloidin with a towel.

Mount in gum damar.

Nuclei stain dark brown, collagen fibers bright red, muscle, red cells, and other tissues a transparent yellow.

Elastic Tissue Stain of Frozen Sections, Krajian[1]

Reagents.—

Elastic Tissue Stain.—

Hematoxylin	3 gm.
95% alcohol	25 c.c.

Dissolve the hematoxylin thoroughly.

Ferric chloride	3 gm.
Ferric ammonium sulphate	3 gm.
Potassium iodide	3 gm.
Distilled water	25 c.c.

Dissolve the chemicals in the distilled water.

Mix the two solutions together. Ready for use.

Acid Alcohol.—1% hydrochloric acid in 70% alcohol.

10% Citric Acid.—

Dissolve 10 gm. of citric acid, c.p., in enough distilled water to make a total of 100 c.c. This solution may develop a growth of molds, in which case a fresh solution must be made.

Saturated Aqueous Solution of Picric Acid.—

Dissolve 1.22 gm. picric acid in 100 c.c. distilled water, with a little heat. Filter when cold.

Van Gieson Stain.—

Saturated aqueous solution of picric acid	100 c.c.
1% aqueous solution of acid fuchsin	5 c.c.

This solution keeps well. If it becomes weak upon long standing, add a few drops of fresh acid fuchsin solution.

Creosote-Isopropanol Mixture.—

Add 50 c.c. of anhydrous isopropanol to
50 c.c. beechwood creosote. Be sure all glassware used is dry.

Isopropanol, xylol, and **gum damar** are also used in this stain.

Technic.—

Cut frozen sections in the usual manner as directed on page 2103.

Place sections on slide under thin celloidin as directed on page 2104.

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[1] Krajian, A. A., and Gradwohl, R. B. H.: Histopathological Technic, St. Louis, 1952, The C. V. Mosby Co.

Staining solutions are in staining jars.

Stain for 3 to 5 minutes in the elastic tissue stain.

Dip in and out of acid alcohol, turning the slide frequently for even decolorization, until the background clears.

Wash in tap water for a few seconds.

Decolorize in 10% citric acid for a few seconds, up to 2 minutes.

Dip in tap water.

Dehydrate quickly in isopropanol from a drop bottle. If the celloidin comes loose at this step, dip once more into the celloidin and dehydrate with anhydrous isopropanol from a drop bottle.

Dip in and out of van Gieson stain about 10 to 15 times, or until the section takes on a good counterstain.

Wash rapidly in a large container of tap water, then blot with several thicknesses of Whatman No. 1 filter paper.

Dehydrate with isopropanol.

Differentiate in equal parts of creosote and isopropanol for 2 minutes.

Place in xylol to clear. Remove excess celloidin with a towel.

Mount in gum damar.

Elastic tissue is black. Amebae stain a pale red with brown nuclei. Connective tissue is dark red.

Weigert's Elastic Fiber Stain[1]

Reagents.—

Weigert's Elastic Fiber Stain.—

Mix and dissolve 2 c.c. of carbolic acid and
1 gm. of basic fuchsin
in 100 c.c. of distilled water, and bring the mixture to a boil. When boiling, gradually add 12.5 c.c. of 30 per cent ferric chloride. A precipitate forms; to render the precipitate more granular, continue the boiling at least 5 minutes.

Cool and filter. Wash the precipitate on the filter paper with distilled water until no more color is seen. Dry the precipitate in the paraffin oven at 55° C. and keep this dry powder in a bottle for making up the staining solution.

Weigh 0.75 gm. of dry powdered precipitate and dissolve in 100 c.c. of 95 per cent alcohol. Boil the mixture in a flask, plugged with cotton wool, in a water bath for 20 minutes. Then cool, filter, and add 2 c.c. of concentrated hydrochloric acid.

Technic.—

Fix tissues in formaldehyde or alcohol. Zenker-fixed tissues stain slowly, and there is greater tendency to diffuse coloring of collagen fibrils. Embed tissues in paraffin or celloidin, or cut frozen sections from formaldehyde-fixed tissues.

Stain sections 20 minutes to 1 hour in Weigert's elastic fiber stain.

Wash in 95 per cent alcohol.

Blot with filter paper and add xylol quickly.

Repeat the blotting, followed by xylol 2 or 3 times, until the section is cleared.

Mount in gum damar.

The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine, before or after staining of the fibers. Carmine stain is difficult to obtain after Zenker's fixation. A light nuclear stain, with some alum hematoxylin, after the fibers have been colored, is preferable. This is a very useful and permanent stain.

Weigert Elastic Stain Method, Raubitschek[2]

Basic fuchsin	2 gm.
Resorcin	4 gm.
Distilled water	200 c.c.

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[1] Krajian, A. A., and Gradwohl: Histopathological Technic, St. Louis, 1952, The C. V. Mosby Co., p. 131.

[2] Personal communication by Dr. Hugh V. Raubitschek, formerly Professor of Pathology, University of Vienna, Austria.

Boil. While briskly boiling, add 25 c.c. of 10 per cent ferric chloride and boil again for 2 to 5 minutes. A precipitate forms. Cool and filter. Discard the filtrate. Dry the precipitate on the filter paper. Put the paper with the dry powder in a beaker with 200 c.c. of 95 per cent alcohol and heat carefully on a waterbath. Fish out the paper, cool, and filter. Add alcohol to the filtrate to make up to 200 c.c. Add 4 c.c. of hydrochloric acid. Since the staining power of every batch is different, it is well to test the stain before using on a specimen and record the staining time on the label of the bottle.

Masson's Trichrome Stain, DeCoursey[1]

Reagents.—

Weigert's Hematoxylin.—

Solution A:

Hematoxylin	1.5 gm.
95% alcohol	100 c.c.

Solution B:

Iron chloride, 29% aqueous solution	4 c.c.
Distilled water	95 c.c.
Hydrochloric acid	1 c.c.

For use, mix equal parts of solutions A and B.

Ponceau-Acid Fuchsin Mixture, Krall.—

Ponceau de xylidene (Krall-Eimer and Amend, N. Y.)	Approximately	0.9 gm.
Acid fuchsin	Approximately	0.1 gm.
Glacial acetic acid		1 c.c.
Distilled water		100 c.c.

Phosphomolybdic Acid Solution.—

Phosphomolybdic acid	1 gm.
Distilled water	100 c.c.

Aniline Blue Stain.—

Aniline blue	2 or 3 gm.
Boiling distilled water	100 c.c.

Add 2 or 3 gm. of aniline blue to 100 c.c. boiling distilled water. Boil for a while longer to dissolve the dye.

Add Glacial acetic acid ---------- 2.5 c.c.

Cool and filter.

Fixation.—

Bouin fixative is preferred, but any fixative may be used.

Carry paraffin sections through xylol and the decreasing strengths of alcohol in the usual manner, to distilled water, then immerse overnight in Bouin solution. This overnight immersion is carried out even with Bouin-fixed tissue.

Just before staining, wash in running water for 20 minutes to remove the picric acid.

Staining.—

After soaking paraffin sections in Bouin's solution overnight and washing as directed above, stain as follows:

Weigert hematoxylin, freshly mixed ----------5 to 10 minutes.
Rinse quickly in distilled water. Tap water causes a precipitate.
Differentiate in 1% acid alcohol under a microscope.
Wash in tap water.
Ponceau-acid fuchsin mixture ----------5 minutes.
Rinse in distilled water.
Phosphomolybdic acid solution ----------2 hours or longer.

This acid decolorizes the collagen and prepares the collagen for aniline blue.

Without rinsing, immerse in aniline blue or light green.

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[1] Simmons, J. S., and Gentzkow, C. J.: Laboratory Methods, Lea and Febiger, 1944.

Leave for 5 to 10 minutes or longer until a deep color is obtained.

Rinse in distilled water.

Return to phosphomolybdic acid 2 to 5 minutes.

This step is important; it removes the blue from the cytoplasm. If light green is used, omit this step.

Rinse with distilled water.

1% acetic acid (in a Koplin jar) 2 to 5 minutes.

This eliminates the phosphomolybdic acid and sets the acid red and blue.

Dehydrate in the usual manner.

Clear with toluene or xylol.

Mount in Canada balsam or gum damar. Just before mounting, pour on a few drops of salicylic acid-toluene or -xylene. The salicylic acid acidulates the balsam and prevents the acid colors from fading.

Aniline Blue Stain of Frozen Sections, Krajian[1]

(For Connective Tissue and Hyaline)

Reagents.—

Mordant.—

Thorium nitrate	2 gm.
Saturated aqueous solution of picric acid	30 c.c.
Acetone	15 c.c.

Dissolve the thorium nitrate in the picric acid solution, then add the acetone.

Azocarmine-Fuchsin Stain.—

Azocarmine solution	1 part
Acid fuchsin, 2 per cent	1 part

Add phenol in 5 per cent proportion (5 c.c. for every 100 c.c. of mixture).

The azocarmine solution is made by dissolving 1 gm. of azocarmine in 100 c.c. distilled water, heating the mixture for 15 minutes, then cooling. Filter and acidify with 1 c.c. of glacial acetic acid.

3% Stannous Chloride.—

Dissolve 3 gm. stannous chloride in enough distilled water to make 100 c.c. of solution.

10% Phosphotungstic Acid Solution.—

Dissolve 10 gm. of phosphotungstic acid in enough distilled water to make a total of 100 c.c. of solution.

Aniline Blue Solution.—

Aniline blue (water soluble)	0.5 gm.
Acetic acid, glacial	8.0 c.c.
Orange G	2.0 gm.
Distilled water	100.0 c.c.

Dissolve with heat. Filter after cooling.

Clearing Agent.—

Creosote (beechwood)	1 part
Xylol	1 part

Technic.—

Fix tissues in 10% Formalin in the usual manner and freeze and cut according to the technic on page 2103. Sections are 7 to 10 microns thick.

Place in mordant for 5 minutes.

Rinse rapidly in tap water.

Transfer to a glass slide as directed in the routine staining method.

Blot under several thicknesses of Whatman No. 1 filter paper.

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[1] Krajian, A. A.: Personal communication.

Dehydrate for a few seconds with anhydrous isopropanol.

Blot again and dip once in thin celloidin (page 2104). Blow over surface until dry.

Rinse in tap water and stain for 5 minutes in the azocarmine-fuchsin stain in a jar.

Wash thoroughly in tap water.

Treat with 3% stannous chloride for 3 minutes.

Rinse and differentiate for 3 to 5 minutes in a 10% phosphotungstic acid solution.

Rinse in tap water.

Stain for 10 to 12 minutes in aniline blue solution.

Rinse in tap water rapidly and thoroughly.

Treat for 2 minutes with 3% stannous chloride solution.

Wash and dehydrate with 3 applications of isopropanol for a few seconds of each application.

Differentiate and clear for 3 minutes in the clearing agent.

Clear for 2 minutes in xylol.

Remove excess celloidin from edges of slide with a towel and mount in gum damar.

Collagen fibers stain deep blue; reticulum, light blue; hyaline, bright red; nuclei, red; erythrocytes, golden yellow; elastic fibers, dark red.

Caution: Keep the solutions in tightly stoppered bottles. They may be used a dozen times.

Staining of Reticulin Fibers in Frozen Sections
Krajian Method

For the demonstration of reticulum and collagen fibers, numerous methods have been devised, but unfortunately most of the methods require paraffin embedding. The paraffin method not only delays the results but may in some manner obscure some of the reticulin fibers which might be demonstrated if the frozen section technic were used.

Morhardt and Krajian[1] have offered an argument in favor of a more detailed study of reticulum or the reticulin fiber network in tissues for the purpose of differentiating various types of neoplasm. They are convinced that any undifferentiated cellular tumor which is without obvious characteristics is incompletely examined unless a reticulum stain is performed. They believe that carcinoma is never productive of an increase in reticulum and that all sarcomas provoke an increase in reticulum. Benign tumors show no changes in reticulum. In their experience with tumors with increased reticulum, they offer the following list: testicular seminoma; pleural mesothelioma; periphery of glioblastoma; spinobasilar chordoma; periphery of astrocytoma; plasma cell myeloma; periphery of metastatic melanblastoma in brain; leiomyosarcoma; leiomyoblastoma; reticulum cell sarcoma; osteosarcoma; lymphosarcoma; retroperitoneal sarcoma, neurogenic; giant cell tumor, osteoclastoma; neurofibrosarcoma; testicular lymphosarcoma; monocytic leukemia (liver); plasma cell leukemia (liver); Wilms' tumor (sarcomatous component); and malignant lymphoma.

Those with normal reticulum are as follows: carcinoma of lung, primary; fibroadenoma of breast; neuroma; uterine adenomyosis; hemangioma; lipoma; neurilemoma; dysgerminoma (ovarian seminoma); carcinoma of thyroid gland; adamantinoma; carotid body tumor.

Tumors with diminished reticulum, that is, displacement of normal tissue, are as follows: hepatoma; carcinoma of ovary; carcinoma of testis; neuroepithelioma; glioblastoma, central portion; glioma, central portion; chorioepithelioma; carcinoma adrenal, metastatic; medulloblastoma (center area);

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[1] Morhardt, R. P., and Krajian, A. A.: Atlas of Tumor Reticulum—An Aid in the Identification of Undifferentiated Cellular Neoplasm. Los Angeles, 1947, Mac Printing Company.

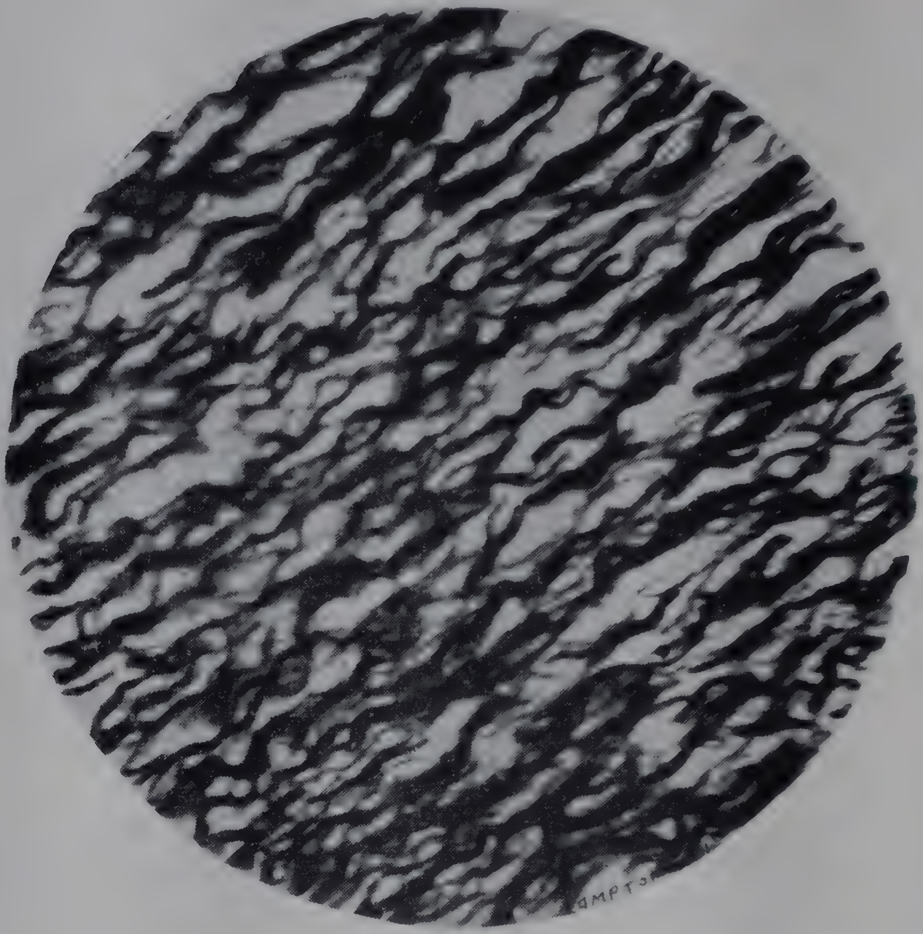

Fig. 628.—Leiomyosarcoma, reticulum stain, Krajian method, high power.

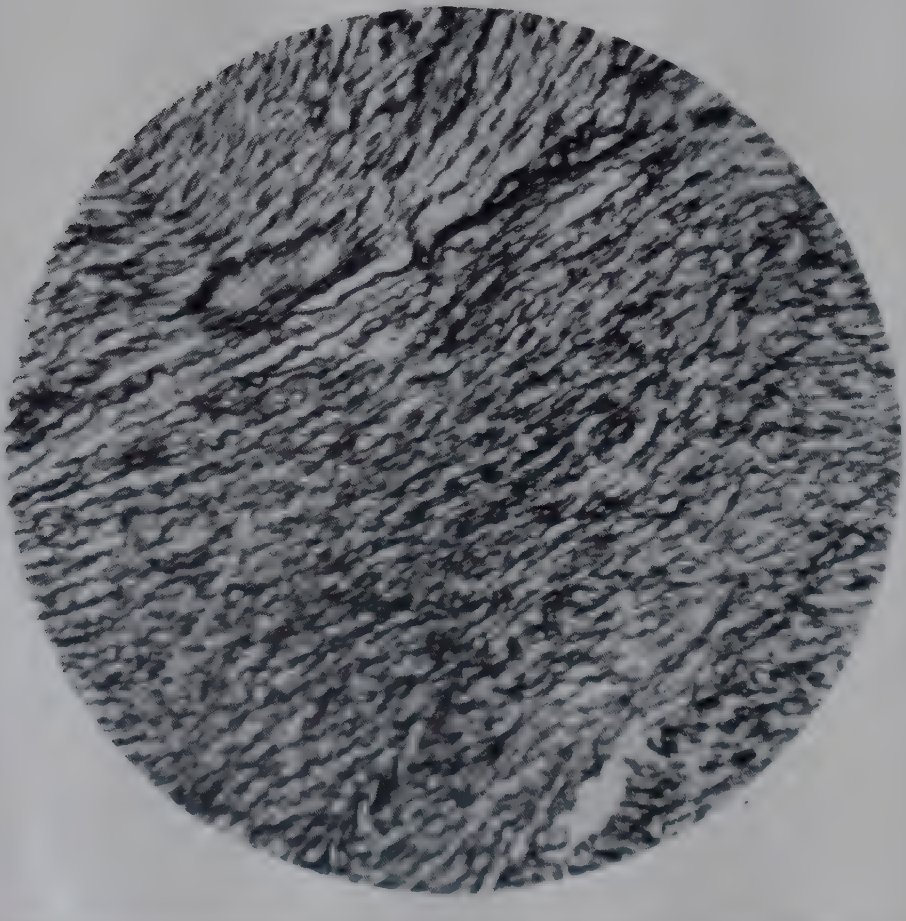

Fig. 629.—Leiomyosarcoma, reticulum stain, Krajian method, low power.

malignant melanoma, lymph node; adrenal cortical adenoma; Ewing's tumor, lymph node; carcinoma pituitary, metastatic; cystic disease of breast; carcinoid in liver; carcinoma of stomach.

Reagents.—

Creosote-Xylol Mixture.—

Add 2 parts of xylol to 1 part of creosote.

10% Formalin.—See page 2102.

10% Ammonium Hydroxide Solution.—

Dilute 10 c.c. of ammonium hydroxide, c.p., to 100 c.c. with distilled water.

0.25% Potassium Permanganate Solution.—

Dissolve 0.25 gm. potassium permanganate, c.p., in 100 c.c. distilled water.

3% Oxalic Acid Solution.—

Dissolve 3 gm. oxalic acid in 100 c.c. distilled water.

Ammoniacal Silver Solution.—

Place 10 c.c. of 10% silver nitrate solution in a Stender dish. Add concentrated ammonium hydroxide solution drop by drop while agitating the container or while stirring with a glass rod until the precipitate disappears.

20% Formalin.—

Dilute 20 c.c. Formalin (40% formaldehyde) to 100 c.c. with water.

0.5% Gold Chloride Solution.—

Dissolve 0.5 gm. gold chloride, reagent grade, in 100 c.c. distilled water.

5% Sodium Thiosulphate Solution.—

Dissolve 5 gm. sodium thiosulphate, c.p., in 100 c.c. distilled water.

Technic.—

Cut frozen sections 7 to 10 microns thick.

Place in a warm 10% ammonium hydroxide solution in the paraffin oven at 56° C. for 10 minutes.

Wash rapidly in distilled water.

Place sections in 0.25% potassium permanganate solution for 10 minutes.

Wash in distilled water and decolorize in 3% oxalic acid until most of the brown color is removed.

Wash in distilled water.

Place in warm ammoniacal silver solution and place in paraffin oven for 40 minutes at 56° C.

Wash in distilled water and place in a 20% warm Formalin solution for 3 minutes (for reducing).

Wash in distilled water and place in 0.5% gold chloride solution for 3 minutes.

Wash in distilled water and place in 5% sodium thiosulphate solution for 5 minutes.

Transfer to glass slide and dehydrate with two applications of anhydrous isopropanol or absolute ethyl alcohol.

Blot twice and dip in thin celloidin, blow over the surface to dry the section, and blot again.

Dehydrate twice with anhydrous isopropanol or absolute ethyl alcohol, and apply creosote-xylol mixture for 3 minutes.

Clear in xylol for 3 minutes and mount in Permount or gum damar.

Reticulin fibers stain black.

Romanowsky Staining of Tissues With Buffered Solutions

The Romanowsky staining of tissue sections has been unsatisfactory because of the necessity of individual regressive differentiation of each section with alcohol, colophonium alcohol, or dilute acetic acid to the desired point. It is often necessary to control this differentiation under the microscope.

Lillie and Pasternack[1] during the course of staining a batch of sections with the MacNeall-French tetrachrome Giemsa modification in 1931 noticed well-stained specimens without any necessity of further differentiation. This accident was found to be due to an unusually acid sample of distilled water. This experience led them to try the effect of various buffer mixtures on stains of the eosin-polychrome-methylene-blue class, and the following procedure was worked out:

Reagents.—

Eosinate of Polychrome Methylene Blue.—

Dissolve 1 gm. eosinate of polychrome methylene blue, prepared by the Balch silver oxide method (10 gm. eosin Y, 9 gm. methylene blue)
in 25 c.c. anhydrous glycerin
and 75 c.c. methyl alcohol, chemically pure.

Phosphate Buffer pH 5.3.—

M/15 Na_2HPO_4	26 c.c.
M/1 NaH_2PO_4	65 c.c.
Distilled water	909 c.c.

Buffered Stain for Use.—

Eosinate of polychrome methylene blue	2 c.c.
Phosphate buffer pH 5.3	50 c.c.

Technic.—

Dehydrate paraffin sections of formaldehyde-fixed or Orth-fixed material in the usual way, down to distilled water.

After the distilled water step, stain for 2 hours in the buffered eosinate of polychrome methylene blue solution.

Rinse in distilled water.

Dehydrate with acetone.

Clear in xylol.

Mount in liquid petrolatum, heavy, U.S.P.

Seal with a pyroxylin cement.

Staining for shorter periods than two hours with the same or more concentrated or heated solutions has not given as satisfactory differential staining. Staining overnight in a solution containing 1 c.c. of stock stain to 50 c c. of buffer is very satisfactory and possibly slightly more differential than the two-hour period, but the greater time requirement is an objection.

When it is especially desired to bring out such tissue elements as red corpuscles and eosinophilic leukocytes, a buffer of pH 4.8 may be employed, but nuclear staining is not as satisfactory as at pH 5.3. On the other hand, a buffer of pH 5.6 stains nuclei and bacteria more deeply, but suppresses eosinophiles and red corpuscles. Basophilic cells are satisfactorily demonstrated at from pH 5.3 to 4.8 and are more conspicuous at the more acid level. Mucin is better shown with less acid buffers.

With a pH 5.3 phosphate buffer, red corpuscles are orange red; eosinophile granules, red; nuclei and tigroid granules, blue; muscle and connective tissue, pink; glia and nerve fibrils, faint pink; pancreatic secretion granules, red; liver cell cytoplasm, light blue; necrotic cells, pink, and bacteria and Rickettsiae, blue. The method has proved particularly satisfactory for the study of tissues of the central nervous system.

Giemsa Staining of Tissues

(Schilling Method, Modified by Gradwohl)

Susa Fixative.—

Mercuric chloride	4.5 gm.
Sodium chloride	0.5 gm.
Trichloroacetic acid	2.0 gm.

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[1] Arch. Path. **14**: 515, 516, 1932.

Glacial acetic acid	4.0 c.c.
Formalin	20.0 c.c.
Distilled water	80.0 c.c.

Fixation, Dehydration, and Embedding.—

Immerse the tissue in Susa fixative for 5 to 48 hours, depending on the size.[1]

Wash in tap water for three to five hours, changing the water frequently.

Place in 50% alcohol for half a day, changing the alcohol several times.

Transfer to 70% alcohol (several changes) for half a day.

Transfer to 95% alcohol for half a day, changing the alcohol several times.

Place in absolute alcohol not longer than one hour. (The sections may be left in this alcohol, if they are very large, for a longer period.)

Clarify in neutral xylol. The tissue must become transparent.

Place the tissues in a mixture of equal parts of paraffin and xylol at a temperature not to exceed 37-40° C. over night. Use special embedding paraffin with a melting point of 56-58° C. Tissuemat or Histowax may be used.

Keep the paraffin oven at a temperature two degrees above the melting point of the paraffin.

Place the tissues in pure paraffin or Tissuemat or Histowax in the paraffin oven for 4 hours.

Transfer to fresh paraffin or Tissuemat or Histowax in the paraffin oven for 2 to 4 hours.

Embed in fresh paraffin or Tissuemat or Histowax in the usual manner.

Staining.—

Cut the sections and mount on slides using Mayer's albumin fixative.

Allow to dry thoroughly.

All solutions for staining sections should be in staining jars.

Place the slides in xylol for 5 minutes to remove the paraffin.

Carry through

Absolute alcohol	1 minute
95% alcohol	1 minute
80% alcohol	1 minute
70% alcohol	1 minute

Place in iodine-alcohol (100 c.c. 70% alcohol and 1 c.c. tincture of iodine) for one to two hours, or until the tissues become a brown color.[1] This removes the mercury.

Treat briefly with 0.2% sodium thiosulphate solution not longer than 10 to 15 minutes, or until the brown color is removed.

Wash for 30 minutes in a jar of tap water.

Wash for 30 minutes in a jar of distilled water.

Dilute Giemsa stain by adding 1 drop of Giemsa stain to each c.c. of neutral distilled water, shaking briefly after the addition of each drop. Avoid shaking too vigorously.

Stain for 30 minutes on a staining bridge, flooding the slide with Giemsa stain.

Renew the stain at the end of the 30 minute staining period, diluting a fresh supply for the re-staining process. Re-stain 30 minutes.

Wash briefly in a jar of distilled water.

Place the slide under the low power objective of the microscope and decolorize as follows:

Decolorization, Dehydration, and Clarification.—

Carried out under microscopic control. This requires very rapid handling.

Avoid over-decolorization.

Use a weak acetic acid solution for decolorizing. This is made by adding a few drops of 1% acetic acid to 50 c.c. distilled water.

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[1] If Formalin-fixed tissues are used, omit the iodine-alcohol-thiosulphate steps in the staining process.

Have on hand a jar of slightly alkaline water, made by adding 2 or 3 drops of 1% potassium carbonate solution to 100 c.c. tap water.

Place the Giemsa-stained slide under the microscope, low power magnification.

Flood the slide with the weak acetic acid solution, and *as soon as* the pink color of the eosin is observed, place the slide in the jar of slightly alkalinized water. The rapidity of this change is very important, because the section will continue to be decolorized as long as the acid remains not neutralized on the tissue.

Dehydration is carried out in staining jars. The solutions are as follows:

1. Xylol	5 parts
Acetone	95 parts
2. and 3.	
Xylol	30 parts
Acetone	70 parts

Place the slide in solution No. 1 for about 5 to 10 seconds,
in solution No. 2 for about 5 to 10 seconds,
in solution No. 3 for 10 seconds or more.

Place in xylol. If the section does not become transparent, place again for a few seconds in solution No. 3 then back in xylol. Continue this process until the tissue is clear, then leave in xylol for several minutes. (Keep the acetone solutions tightly stoppered to prevent absorption of undue amounts of moisture.)

Mount in gum damar.

Butyl Alcohol in Dehydration and Clearing Specimens That Have Been Embedded in Paraffin (Zirkle[1])

Zirkle[1] describes the use of butyl alcohol in dissolving some of the superfluous xylol which adheres to the slides and is carried on them into the absolute alcohol. This method prevents a milky precipitate which is commonly seen.

Water	1 part
Xylol	1 part
N-butyl alcohol	1 part
Ethyl alcohol	2 parts

The ethyl alcohol-butyl alcohol mixture dissolves both water and xylol, keeps them in solution at the same time, and forms a single clear liquid. Use for cleaning slides to remove both water-soluble (glycerin, glucose, etc.) and fat-soluble material (immersion oil, balsam, etc.). Dilute ammonium hydroxide may be substituted for the water and carbon tetrachloride for the xylol. This combination forms a potent cleanser.

A very useful addition to cytologic technic is the application of n-butyl alcohol in the hydration and dehydration of cut sections. It has often been observed in staining paraffin sections that carrying the slides through xylol, then absolute alcohol, then decreasing strengths of alcohol results in clouding of the section because of the small amount of xylol which remains after the first step. Some xylol is also carried to the alcohols, so that when they come in contact with the water, clouding occurs. The following series will serve to eliminate such cloudiness and precipitation:

1. Xylol 100%.
2. Xylol 95%, n-butyl alcohol 5%.
3. Absolute ethyl alcohol 90%, n-butyl alcohol 10%.
4. Absolute ethyl alcohol 100%, etc.

Zirkle states that he has often passed more than a hundred slides through a single series of Koplin jars without a precipitate and without renewing any of the solutions.

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[1] Science, p. 481, Nov. 23, 1934.

VAGINAL SMEARS IN THE DIAGNOSIS OF UTERINE CANCER

The vaginal smear method in diagnosis of uterine cancer has become a valuable laboratory procedure. According to Meigs,[1] delay is responsible for a majority of deaths from cervical cancer, a delay to which both patient and physician contribute. He used a simple method described by Papanicolaou[2, 3, 4] for making a presumptive diagnosis of uterine carcinoma. With this method a diagnosis of uterine carcinoma may be made by examination of the vaginal smear. In Meigs's report, 200 women were studied, using the technic described by Papanicolaou.[2]

PAPANICOLAOU TECHNIC[2]

Attach a slightly curved glass pipette, 8 inches long, to a 3-inch rubbed suction bulb. The pipette must be absolutely dry, and the patient must not have douched that same day. These precautions are necessary since water in the vaginal smear destroys the cellular detail.

Compress the rubber bulb and then insert the pipette into the vagina as far as possible in order to reach the fornix.

Release the bulb and withdraw the pipette slowly. Blow the material on a previously marked slide and drop the slide immediately into a bottle of fixative. The fixative is made by mixing equal parts of ether and 95% alcohol.

Since drying destroys cellular detail it is essential that the slide be placed immediately in the fixative. Fixation for 15 minutes is sufficient, but slides may be left in the fixative without deterioration for as long as 2 weeks, and 1 hour is suggested as a routine fixing period.

Reagents.—

Harris Hematoxylin.—

Prepare Harris hematoxylin in the usual manner (page 2105) but omit the acetic acid.

Dilute with an equal volume of distilled water.

Filter through filter paper.

From time to time, as it is in use, add more Harris hematoxylin in order to maintain standard staining results.

0.25% Hydrochloric Acid Solution.—

Add 0.25 c.c. of concentrated hydrochloric acid, c.p., to distilled water, and dilute to 100 c.c. with distilled water.

OG 6.—

0.5% alcoholic solution of orange G	100 c.c.
Phosphotungstic acid	0.015 gm.

EA 36.—

Light green SF yellowish, 0.1% alcoholic	45 c.c.
Bismarck brown, 0.5% alcoholic	10 c.c.
Eosin Y, water and alcohol-soluble, 0.5% alcoholic	45 c.c.
Phosphotungstic acid	0.2 gm.
Lithium carbonate, saturated aqueous solution	1 drop

NOTE: All stains are certified stains.

Alcohol-Xylol Mixture.—

Equal parts of absolute alcohol and xylol.

EA 50.—

This is a stain comparable to EA 36.*

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*Obtainable from Ortho Pharmaceutical Corporation, Raritan, N. J.

[1] Surg. Gynec. & Obst. 77: 449, 1943; Lab. Digest 8: 2-6, Jan., 1945.

[2] Papanicolaou, G. N., and Traut, H. F.: Am. J. Obst. 42: 193, 1941.

[3] Idem: New York J. Med. 13: 467, 1943.

[4] Idem: Diagnosis of Uterine Cancer by Vaginal Smear, New York, 1943, Commonwealth Fund.

Technic.—

Carry out the staining process in staining dishes.

After fixation, transfer the slides, without drying, directly from the alcohol-ether mixture to 80% alcohol.

Dip 4 times in 80% alcohol.
Dip 4 times in 70% alcohol.
Dip 4 times in 50% alcohol.
Dip 4 times in distilled water.
Stain for 6 minutes in the Harris hematoxylin (prepared as given above).
Rinse in distilled water.
Dip 6 times in 0.25% hydrochloric acid solution.
Place in running tap water for 6 minutes.
Rinse in distilled water.
Rinse in 50% alcohol.
Rinse in 70% alcohol.
Rinse in 80% alcohol.
Rinse in 95% alcohol.
Stain for 1½ minutes in OG 6.
Rinse in 95% alcohol.
Rinse in 95% alcohol in another staining dish.
Stain in EA 36 for 1½ minutes. (Or use EA 50.)
Rinse in 95% alcohol.
Rinse in 95% alcohol in another staining dish.
Rinse in 95% alcohol in another staining dish.
Rinse in alcohol-xylol.
Rinse in xylol.

Mount in Permount, gum damar, or Canada balsam, or any other satisfactory neutral mounting medium.

The original methods of staining and preparing stains as described by Papanicolaou have been materially changed and much improved by the work of Pharr and others.[1] There has been great difficulty in suspending dry, powdered stains in various solvent agents such as water, alcohol, and glycerin, with resulting variations in weight of stain per volume of stock solution. This has caused difficulties with the employment of cytologic stains used in the Papanicolaou method.

Eosin, light green S.F. yellowish (EA), and orange G are readily soluble in water; this would be the stock solution of choice except for two reasons: the aqueous stock solutions are not sufficiently stable to be of practical value; and these stains tend to lose their sharpness of color when suspended in water. Pharr and associates have found the use of a mechanical stirrer a valuable adjunct in their work. The mechanical stirrer used in their study was the Waring blendor.

STAINING METHOD OF PHARR, WOOD, AND TRAUT[1]

Preparation of the Stain.—

Orange G.—

Heat 380 c.c. of 95% ethyl alcohol to the boiling point and pour sufficient heated alcohol into the stirrer cup to cover the blades. This prevents the powdered stain from adhering to the blades.

Add 1 gm. of orange G (National Aniline R*).

Fill the stirrer cup with the heated alcohol to approximately one-fourth its volume.

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*National Aniline Division, Allied Chemical & Dye Corp., 40 Rector St., New York City.

[1] Pharr, S. L., Wood, D. A., and Traut, H. R.: Am. J. Clin. Path. 24: 2, 1954.

Mix the solution for 1 or 2 minutes, turning the motor off and on several times for maximum mixing.

Pour off the supernatant fluid after allowing the unsuspended stain to settle momentarily.

Add additional alcohol to the remaining undissolved stain and pour off after mixing.

Repeat until all alcohol has been used and all stain is in solution.

Allow the solution to *cool*, and then filter. There will be a slight precipitate of orange G, since the stain is present in excess.

Add 0.4 gm. of phosphotungstic acid

and 3 c.c. of glacial acetic acid to the filtered solution.

The orange G working solution is now ready for use and is stable for one month to six weeks. Store excess working solution in an amber-colored bottle to prolong its stability.

EA Stain.—

Cover the blades of a clean, *dry* mechanical stirrer with a portion of a measured 380 c.c. of 95% ethyl alcohol. Do not heat the alcohol.

Add 2 gm. of eosin (National Aniline R)

and 1 gm. of light green S.F. yellowish (National Aniline R)

and fill the stirrer cup to approximately one-fourth its volume with alcohol. Note that the Bismarck brown has been omitted as a constituent of this stain.

Mix the solutions, as previously described, until all the stain is in solution.

Allow to stand overnight, but DO NOT FILTER.

Add 0.4 gm. of phosphotungstic acid

and 3 c.c. of glacial acetic acid.

The EA working solution is stable for one month to six weeks if it is covered to protect it from deterioration by light or if it is poured back into a dark bottle after use.

It is essential that the stirrer cup be clean and dry to obtain a sharp, bright stain. Spraying from the forceful pressure of the stirrer blades is avoided by placing a filter paper or paper towel under the stirrer cover.

Since these stains are water soluble, the stirrer cup and blades are easily cleaned.

Stains prepared in this manner have been consistent from one batch to another and have not precipitated during use.

Mayer's Hematoxylin.—

Distilled water	1,000 c.c.
Hematoxylin (National Aniline R)	1 gm.
Ammonium or potassium alum	50 gm.
Sodium iodate	0.2 gm.
Citric acid	1.0 gm.
Chloral hydrate	50 gm.

Dissolve the hematoxylin in 1,000 c.c. of distilled water that has been heated to 55° to 60° C., and rotate until all the stain is in solution.

Add the alum and sodium iodate, taking care that the 0.2 gm. of sodium iodate has been accurately weighed.

Rotate until the alum has dissolved.

Add the citric acid and the chloral hydrate. The order of mixing must not be changed.

Allow the solution to stand overnight before use.

This solution is stable for two months if the staining time is gradually increased from an initial 6 minutes to 8 minutes after prolonged use. Occasional filtering may be necessary to remove accumulated cellular debris.

Staining Technic.—

Prepare slides and fix immediately in equal parts of ether and 95% ethyl alcohol for a minimum of 30 minutes.

Immerse in 70% ethyl alcohol for approximately 10 dips, or until glossy.

Immerse in 50% ethyl alcohol for approximately 10 dips, or until glossy.

Wash in *distilled* water for approximately 10 dips, or until glossy. This water should be replaced frequently.

Stain with Mayer's hematoxylin for 6 to 8 minutes, according to the age of the stain.

Wash in a gentle stream of running tap water for a minimum of 20 minutes. If this time is increased to 45 minutes, there will be slightly improved nuclear detail.

Dehydrate in graded ethyl alcohol, 50%, 70%, 80%, and 95%, immersing for 10 dips each.

Stain in orange G for 1½ minutes, agitating intermittently.

Immerse for 10 dips each in 3 changes of 95% ethyl alcohol.

Stain in EA for 1½ minutes, agitating intermittently.

Immerse for 10 dips each in 3 changes of 95% ethyl alcohol.

Dehydrate in absolute ethlyl alcohol 10 dips.

Dehydrate in a mixture of equal parts of absolute ethyl alcohol and xylol, 10 dips.

Clear in xylol, immersing for approximately 10 dips or until glossy.

Mount in gum damar, Clarite, etc.

If it is found that the local tap water used in washing slides following staining with hematoxylin is not sufficiently alkaline, that is, approximately pH 7.4, the lithium carbonate method of alkalinizing must be employed.

Another safeguard is to avoid prolonged standing in the alcohol washes following staining with EA, which results in decolorization of both the eosin and the light green. The stained slides may stand in xylol for short periods without significant effect, but standing several hours in this solution decolorizes the eosin.

Take care to prevent water absorption and evaporation, by keeping the staining dishes covered when not in use.

For staining tissue sections by the Papanicolaou method, remove the paraffin with xylol------------5 minutes.

Wash twice in absolute alcohol.

Dehydrate, beginning with 70% ethyl alcohol, and carry out the usual routine staining procedure.

Increase the hematoxylin staining time to 15 minutes.

It is better to stain paraffin sections on the same day that they are cut. Do not place them on a heated drying rack.

The Ayre Surface Biopsy in the Diagnosis of Uterine Cancer

Ayre[1] modified the original technic of Papanicolaou by taking smears directly from the cervical os. A kind of rubbing technic is particularly useful where there are erosions or visible evidence of pathology around the cervix. He uses a hollowed out end of an ordinary wooden tongue depressor, so that the hollowed out end of this applicator rubs directly over the cervix and procures cells directly from this part. The pointed portion of the other end of the applicator removes malignant cells likely to be found in the mucus at the external cervix portal in cases of cancer. The cytology of the canal is considerably different from that of the vagina. In malignant cases there are fewer squamous epithelial cells and a greater quantity of leukocytes but, more significantly, a greater concentration of malignant cells is to be found. This, Ayre believes, will increase the percentage of positive cases and reduce the percentage of error.

This method is preferable to the ordinary biopsy which carries with it possibility of trauma, bleeding, and distention. If a positive diagnosis is made by vaginal smear, then the biopsy can be made and radium therapy begun. In some cases the presence of sepsis presents a definite contraindication to cervical biopsies and in such cases smears can be taken immediately without stirring up the infection. Ayre found this procedure quite useful in differentiating between a degenerating malignancy and fundal cancer with secondary infection.

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[1] Am. J. Obst. & Gynec. 51: 741, 1946.

Papanicolaou Endocervical and Endometrial Smears

This affords an opportunity to obtain malignant cells coming from the fundus through to the endocervical canal. Papanicolaou[2] states that these malignant conditions within the fundus or cervical canal are highly proliferative. Technic for obtaining this material is to use a small pliable laryngeal cannula attached to an ordinary syringe. Insert this cannula directly within the canal as high up as desired, and pull out the secretion. Eject this upon a glass slide and proceed in the same manner as in fixing and staining vaginal smears.

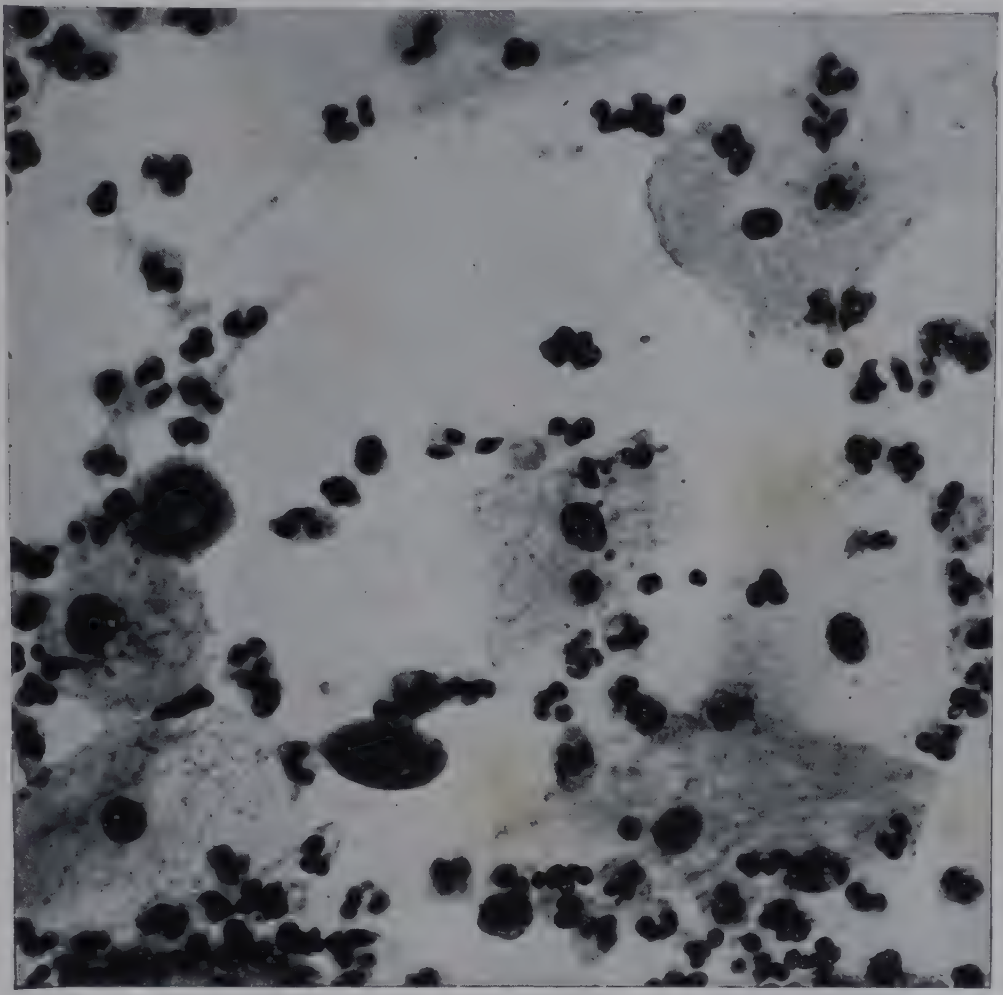

Fig. 630.—Negative smear showing cornified and precornified cells and two basal cells in lower left hand corner (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

DIAGNOSTIC CRITERIA

Meigs emphasized that in order to recognize the atypical cells found in carcinoma of the cervix and endometrium, one must know the *normal cells.* These normal cells may be divided into three groups: the cornified series; the basal cells; and the endometrial cells. Cornified cells are again divided into two groups, precornified and fully cornified. The precornified cells are large waferlike cells with abundant thin green cytoplasm and a vesicular nucleus (Fig. 630). The fully cornified cells are similar to the precornified cells with the exception that the cytoplasm stains pink and the nucleus is pyknotic. The cytoplasm may show some signs of degeneration such as wrinkling and granulation (see Fig. 630).

Basal cells are smaller, round or oval cells with green cytoplasm and a vesicular nucleus (see Fig. 631). With extreme atrophy of the genital tract the nuclei of the normal basal cells may contain chromatin in large clumps. Endometrial cells are the smallest of the normal cells, showing a small vesicular nucleus with very little cytoplasm. They show little variation in size and shape (see Fig. 632).

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[2] Personal statement.

For the diagnosis of *carcinoma of the cervix,* the criteria are as follows: the cells show great variation in size, shape, and form—much greater variation than is usually seen in the pathologic section of the same tumor. The most striking variation in shape appears in the tremendously elongated "tadpole" cells (see Fig. 633), which have a rounded head containing the nucleus and a long thin tail. Long fiberlike cells may be found (see Fig. 634). These cells are extremely elongated, with tapered ends, and contain an elongated nucleus, or two or three nuclei. They may be found singly or in groups.

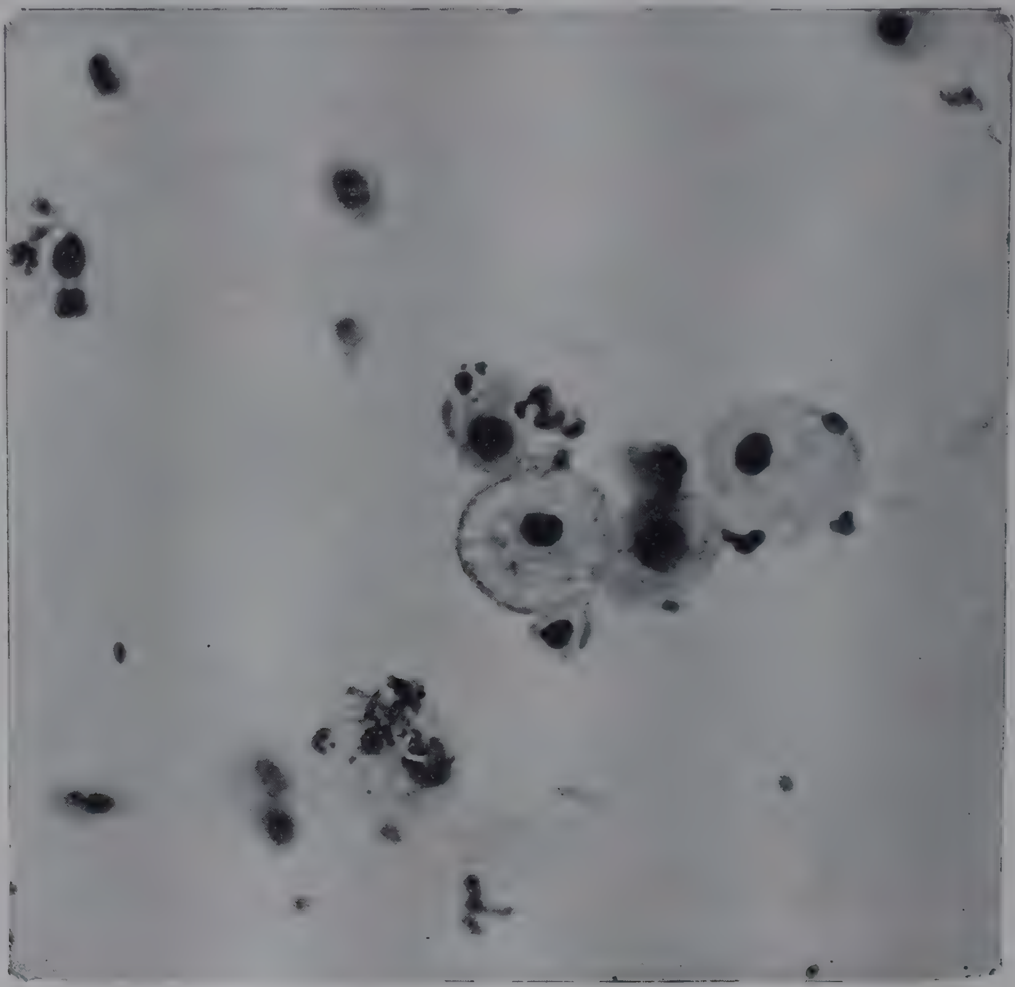

Fig. 631.—Negative atrophic smear showing a field of basal cells (×825). Courtesy Surgery, Gynecology, and Obstetrics.)

Many atypical cells have vacuolated cytoplasm with a foamy appearance, but cellular vacuolization is not diagnosed as malignancy. Staining characteristics of the cytoplasm are not diagnostic, since in the vaginal smear the atypical cells may have either basophilic or acidophilic cytoplasm. This variation in staining is due to differences in the acidity of the vagina as the result of either infection, parasitic infestation, or estrogen elaboration.

The nuclei of abnormal cells show marked variation in shape and size, and bizarre forms occur. Increased activity of the nuclei may be noted, indicated by clumping of the chromatin, granularity of the nucleus, and occasionally an apparent increase in the amount of nuclear material. Nucleoli are seen occasionally but not consistently enough to make observation of the nu-

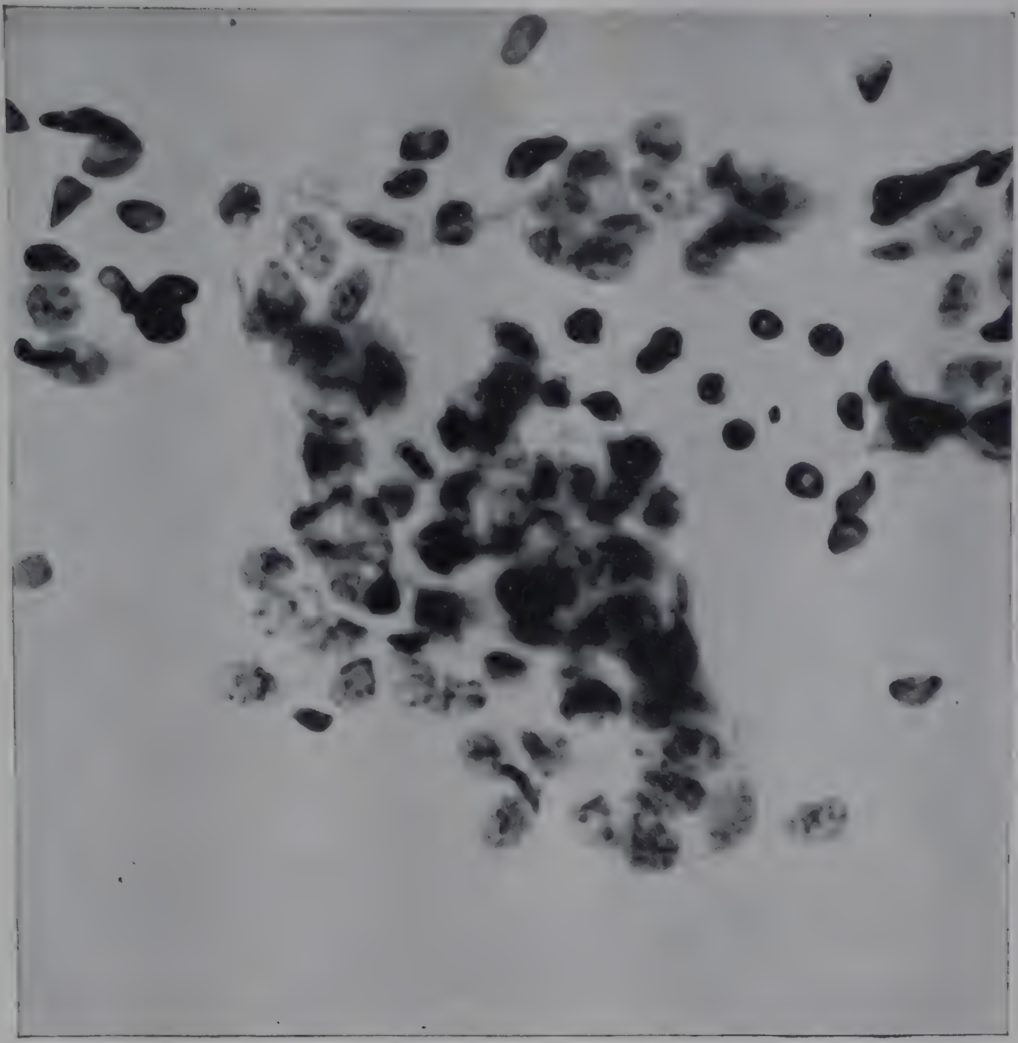

Fig. 632.—Smear taken from normal endometrium after surgical removal of the uterus. (Courtesy Surgery, Gynecology, and Obstetrics.)

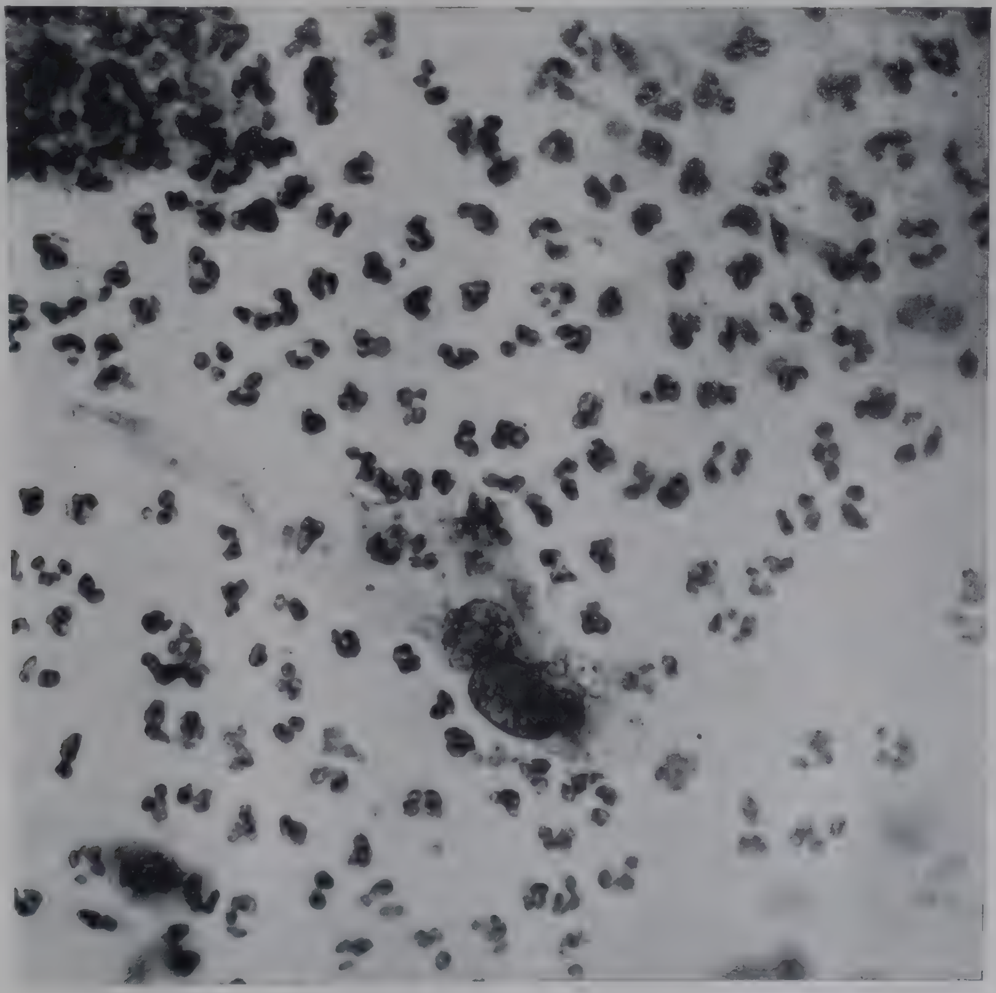

Fig. 633.—Carcinoma of the cervix. A huge bizarre-shaped cell with double nuclei illustrating the marked variation in size and shape of cells encountered in carcinoma of the cervix. ("Tadpole" cell.) (×825.) (Courtesy Surgery, Gynecology, and Obstetrics.)

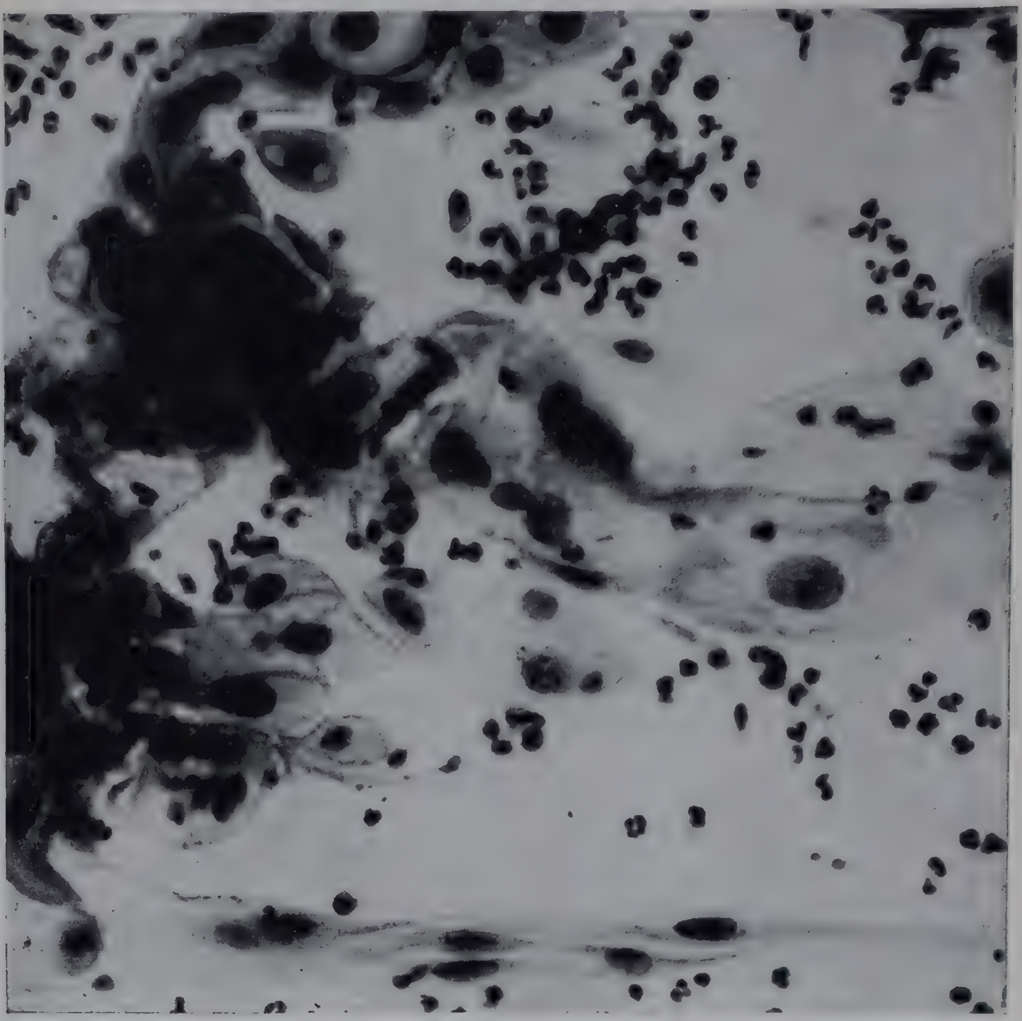

Fig. 634.—A group of atypical cells seen in epidermoid cervical cancer. The long fiber-like cells in the lower portion of the photomicrograph are abnormal cells encountered frequently in positive smears (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

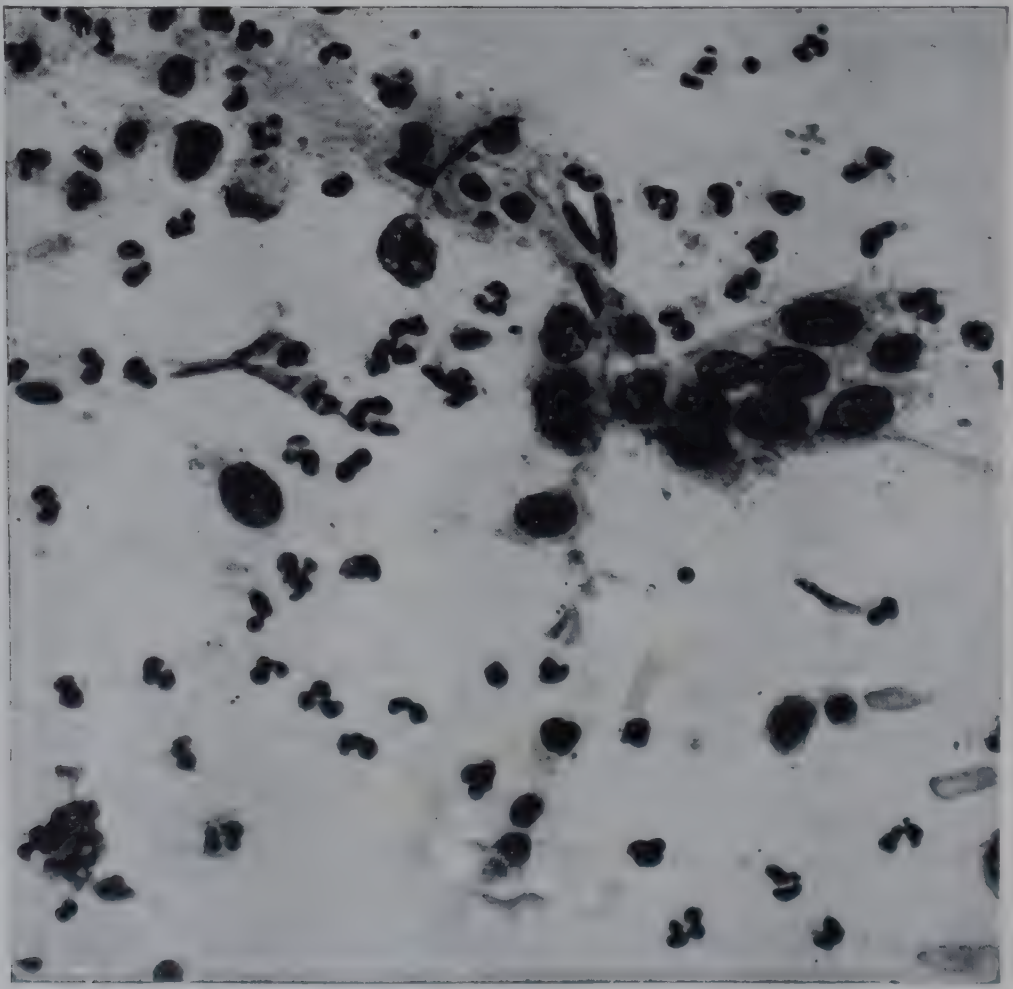

Fig. 635.—Atypical cell group seen in epidermoid carcinoma of the cervix, illustrating the abnormal variation in size and shape of the nuclei (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

cleolus an important aid in diagnosis. Mitoses may occur but are very rare. The most frequent variation of the nucleus is its size. In the abnormal cell the nucleus often fills the entire cell, leaving a thin but distinct rim of cytoplasm (see Fig. 635). Often multinucleated giant cells are seen, occasionally containing as many as a dozen nuclei (see Fig. 636). Histiocytes are often present in positive smears (see Fig. 637). They vary in size. They often contain phagocytosed cellular elements such as red blood cells, leukocytes, and cellular debris. They frequently have acidophilic cytoplasm. Remember that histiocytes may appear in normal smears. Leukocytes are usually present but their numbers vary. Red blood cells are present in every positive smear. Meigs stated that he has never made a diagnosis of malignancy without some evidence of blood.

For the diagnosis of *endometrial cancer,* the same general criteria apply: evidence of blood must be present; an increase in leukocytes is usually seen; histiocytes are frequently found. The variation in size of individual atypical cells in endometrial cancer is not as great as in cervical. The cells are more like those seen in stained sections of the same tumor. Abnormal endometrial cells are usually found in clumps which can be easily seen under low power. The nuclei vary tremendously in size. Haumeder found the nuclei of cells in carcinoma of the endometrium to be twice as large as those found in hyperplasia of the endometrium. Occasionally in endometrial carcinoma large cells are seen with an abundant amount of cytoplasm and large vesicular nuclei (see Fig. 638), but they are rare. Mitoses (see Fig. 639) are very rare.

The authors attempted to determine whether the abnormal cells seen in the vaginal smear were actually tumor cells or whether they represented a tissue reaction secondary to the presence of cancer. Smears taken from the tumor at operation were compared with those made from the vaginal secretion. In seven cases of cervical cancer and in three cases of endometrial cancer, the same types of cell were found in the vaginal secretion as in the smear from the tumor itself.

Important Points.—

It is important when a negative smear is obtained to repeat the examination because one negative smear cannot be depended upon to exclude the presence of cancer. Sixteen smears were taken over a period of seven days from one patient with cervical cancer. Cancer cells appeared in six smears only.

In three cases cancer was indicated by the smear, but curettage proved negative. In another case the smear was positive but no cancer was found on routine pathologic examination after complete removal of the uterus. Serial sections were not made. It must be remembered that failure to demonstrate cancer on routine pathologic examination of the excised specimen does not exclude the presence of cancer. The proof rests upon a negative result following complete serial section of the uterus.

Should operation for uterine cancer be made solely on the evidence of a positive vaginal smear? This question was answered by Meigs in these words, "The positive smears should be confirmed by biopsy or curettage."

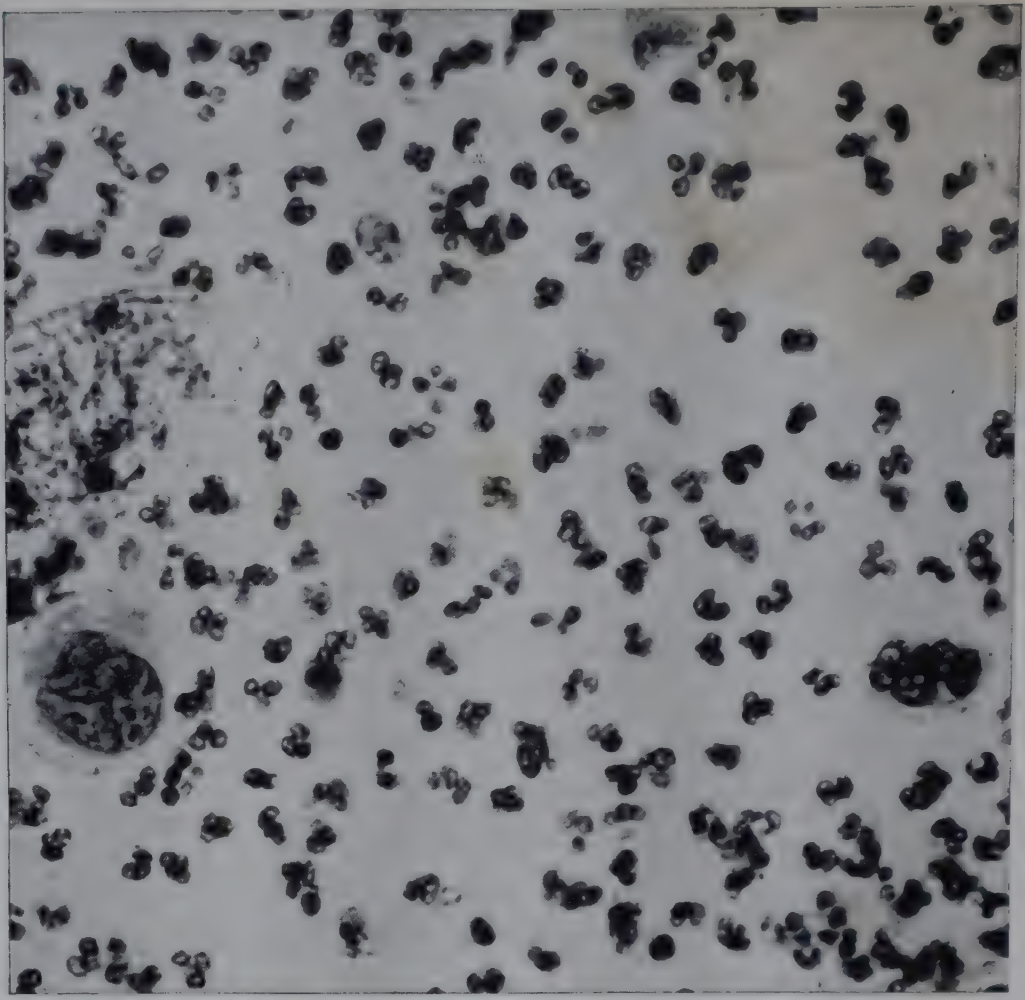

Fig. 636.—Two multinucleated giant cells seen in epidermoid carcinoma of the cervix (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

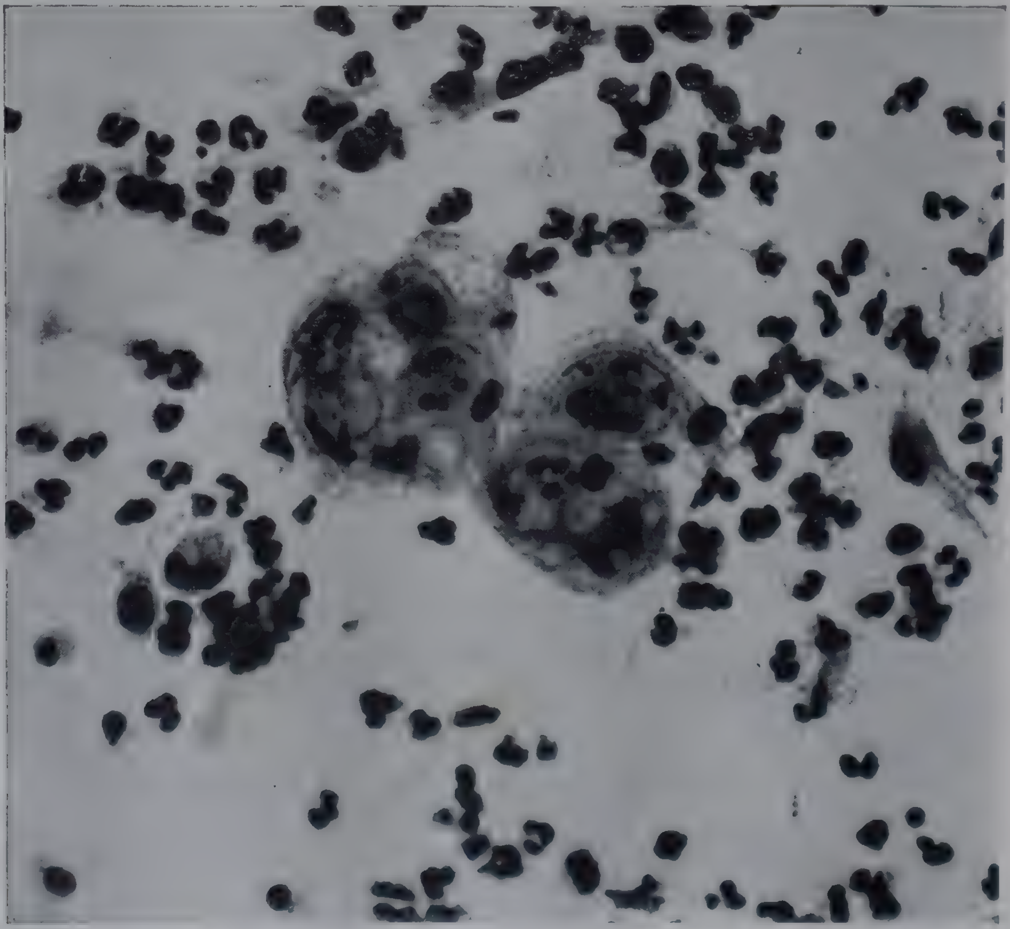

Fig. 637.—Two large histiocytes with phagocytosed polymorphonuclear leukocytes and red blood cells seen in a vaginal smear from a case of epidermoid cervical cancer (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

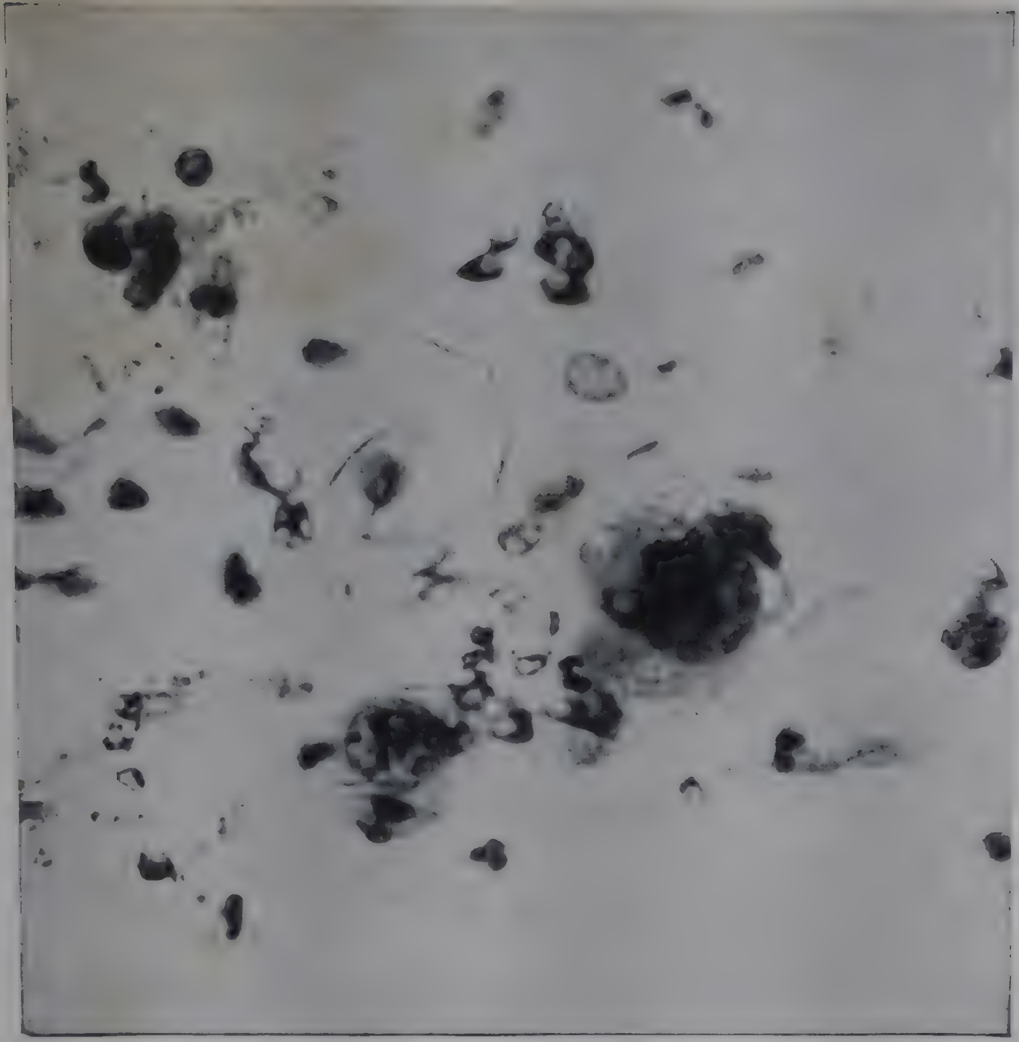

Fig. 638.—Adenocarcinoma of the endometrium. Atypical large cells with huge vesicular nuclei—a rare type of cell seen in endometrial carcinoma (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

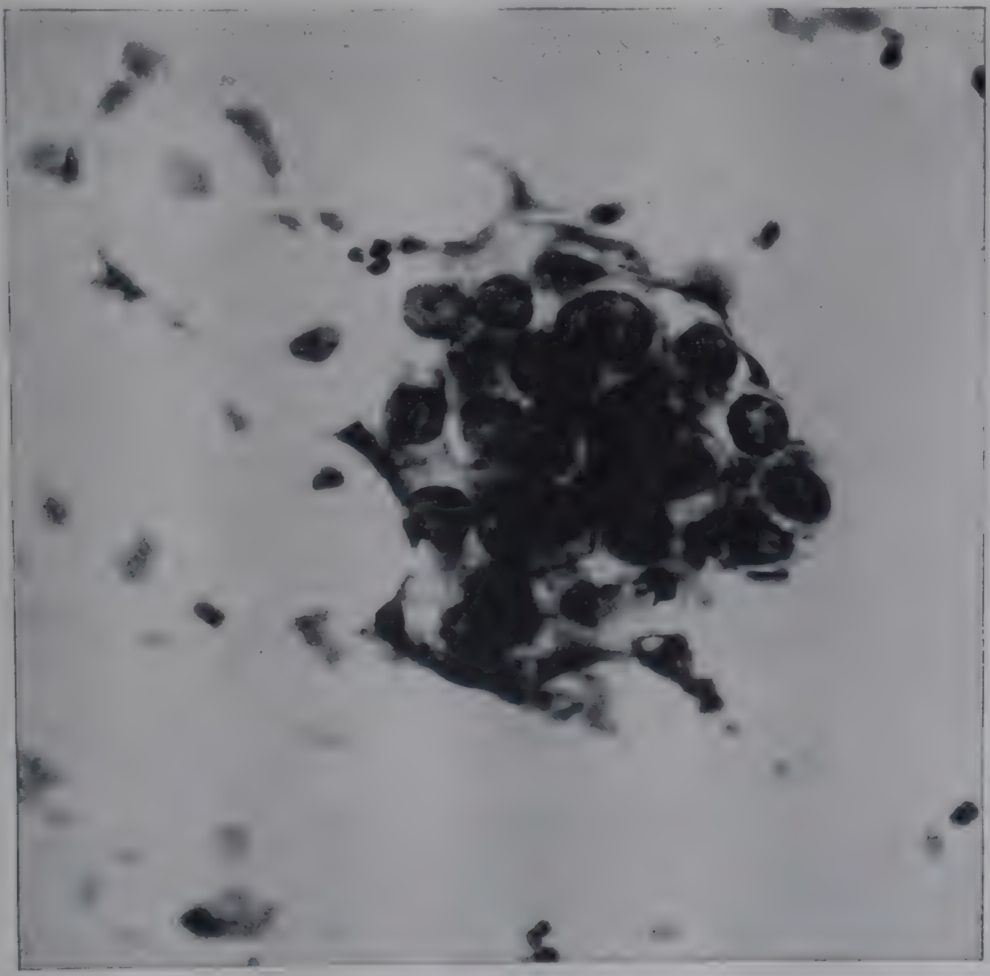

Fig. 639.—Adenocarcinoma of the endometrium, group of cancer cells. One cell is in mitosis (×825). (Courtesy Surgery, Gynecology, and Obstetrics.)

This method should be a part of every routine physical examination in office, outpatient department, and hospital.*

Papanicolaou Technic for Staining Urine Sediment, Gastric and Bronchial Aspirates, Pleural and Peritoneal Fluids, etc., for Malignant Cells.—

Fix the fluid immediately after aspiration in an equal amount of 95 per cent alcohol (50 c.c. of each is usually sufficient).

Centrifuge as soon as possible at medium speed for 20 minutes, and remove the supernatant fluid.

If smears cannot be prepared immediately, cover the sediment with absolute alcohol, and place in the refrigerator. Prepare the smears from the sediment on slides which have been previously coated with a thin film of Mayer albumin fixative. Spread the smear with another slide as evenly as possible. When it begins to dry around the edge but is still moist in the center, fix the slide in alcohol-ether for a minimum of one hour and stain as for vaginal smears.

COLLECTION, PROCESSING, AND STAINING OF SPUTUM FOR CYTOLOGIC DIAGNOSIS[1]

Sputum.—A deep cough specimen is collected into a wide-mouth bottle containing 70% alcohol. Because of the hardening effect of the alcohol, the specimen should be sent to the laboratory and smears made without delay.

Bronchial Aspirants or Washings.—Bronchial secretions are collected at the time of bronchoscopy by aspiration or by lavage into a small-mouthed jar with a physiologic saline solution. Aspirated bronchial secretions or bronchial saline washings are mixed immediately with at least an equal quantity of 95% alcohol. Rinse the collection tube 2 or 3 times with 95% alcohol, and add these washings to the specimen. The type of each specimen and the site at which it was collected should be accurately recorded on each label.

In cases in which secretion is scant, Clerf and Herbut recommend obtaining the material from the smaller bronchi by means of a small gauze spoon affixed to the tip of a carrier. Such material is smeared directly on slides which are fixed immediately in alcohol-ether solution.

Aspiration of tracheal bronchial secretions by catheter is another method reported by Cahan and Farr. This method is the particular one for cases in which adequate ''deep cough'' sputum is not produced or bronchoscopy is for any reason undesirable. It has the additional advantage that it is feasible as an office or clinical procedure.

Papanicolaou Method for Sputum Smears.—

Collect the sputum directly in 70 per cent alcohol.

Spread it on slides which have been coated with albumin. If there are hard masses they may be crushed slightly with another slide.

Fix the smear in alcohol and ether for a minimum of one hour and stain as for vaginal smears.

Caution.—

In the staining methods used for all this material—vaginal smears, cervix biopsy material, endocervical and endometrial secretion, urine, gastric, bronchial, pleural, peritoneal fluids, and sputum—be sure that the smears are never allowed to dry at any time.

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*All cuts by permission of Surgery, Gynecology and Obstetrics.

[1] Papanicolaou: Atlas of Exfoliative Cytology, published for The Commonwealth Fund by Harvard University Press, Cambridge, Mass., 1954.

Preparation of Sputum With Trypsin for Cytologic Study.—

Because of the physical character of sputum, it is often difficult to make sputum examinations according to the method of Papanicolaou. If one examines sputum in the ordinary way, nasal and oral materials are usually present, and the true bronchial sputum is minimal and may be entirely overlooked in the selection of material for screening. It is believed that a negative cytologic report is not justified in the usual test, unless at least fifteen different slides have been studied.

Farber, Pharr, Wood, and Gorman[1] suggested the use of trypsin for its mucolytic and digestive action in the preparation of sputum for cytologic study. Trypsin is chiefly known for its selective enzymatic digestion of nonviable organic material.

Reagents.—

Trypsin Solution.—

Dissolve 125 mg., or 125,000 Armour units, of trypsin
in 12.5 c.c. of Sorensen buffer, pH 7.1.
This amount will liquefy 8 to 10 c.c. of sputum.

Caprylic Alcohol.—

Human Plasma.—

Technic.—

Add the trypsin solution to 8 or 10 c.c. of sputum in a centrifuge tube with a capacity of two or three times the total amount of mixture.

Add 1 or 2 drops of caprylic alcohol, depending upon the agitation time. This reduces surface tension and prevents foaming upon agitation. The time required to liquefy a specimen thoroughly depends upon its initial viscosity, but the most tenacious specimens usually respond within 15 minutes.

The tubes must be simultaneously heated and agitated. Place them in a serologic water bath at 57° C., and force air through. Use a portable compression tank to the faucets of which are attached glass droppers by means of rubber tubing. Insert the droppers into the centrifuge tubes containing the sputum-trypsin mixture. Regulate the pressure to a forceful stream.

After the specimen has been agitated, layer 0.5 to 1 c.c. of human plasma to the bottom of the centrifuge tube, using a pipette or capillary dropper. The plasma not only aids in arresting the digestive process, but also acts as an adhesive agent, replacing the liquefied mucus, and prevents the loss of cells from the slide during fixing and staining.

Centrifuge, after digestion is complete, and collect the sediment on glass slides.

Fix in equal parts of alcohol and ether, and stain in the usual manner by the Papanicolaou method.

Trypsin has a marked effectiveness in reducing the number of mature, nonviable squamous cells, and at the same time it does not alter the staining characteristics of the cellular material, nor does it have any noticeable bad effect on the appearance of malignant cells.

DIAGNOSIS BY CYTOLOGIC STUDY OF BRONCHOSCOPICALLY REMOVED SECRETIONS

The value of examination of bronchoscopically removed secretions in the early diagnosis of bronchogenic carcinoma is set forth fully by Herbut and

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[1] Science **117**: 687, 1953.

Clerf.[1] This method has been used with considerable success in countries other than our own. In England[2] positive diagnoses were reported in as high as 68 per cent of cases of bronchogenic carcinoma, in Russia,[3] 70 per cent, in Denmark,[4] 85 per cent, and equally enthusiastic reports have been published in Latin American literature.[5] As these writers state, this has not been a popular method in this country as witnessed by the following statements: "Only exceptionally may the diagnosis be made from a study of the cytology of the sputum."[6] "The pathologist may on rare occasions make a diagnosis from fragments of tumor discovered in the sputum."[7] "Rarely portions of growth are expectorated and can be submitted to microscopy."[8] "With exception of the two cases (in a series of 175) just mentioned there has been no case in which the examination of the sputum has revealed tumor cells."[9] "Sometimes fragments of tumor tissue acceptable for diagnostic purposes are obtained by bronchial aspiration."[10] "It has been of value in a few of our cases."[11]

Contrasted with these rather discouraging remarks are the enthusiastic words and definite scientific proof found in this article by Herbut and Clerf. In order to obtain reliable material, one must examine bronchial secretions rather than sputum for tumor cells. Sputum is more dilute than are bronchial secretions, and thus the chances of finding cancer cells are considerably diminished. Furthermore, expectoration of sputum does not occur until late in the disease, at which time a diagnosis can be easily made by other means. Bronchoscopy, on the other hand, is performed routinely in all modern clinics in every case of suspected pulmonary carcinoma. One must obtain secretions which come from the region of the suspected tumor for this examination. This material is more likely to contain cancer cells than are the more dilute secretions found in the trachea and the even more dilute sputum.

Material and Method.—

Herbut and Clerf first made a number of experimental observations on cadavers, obtaining bronchial secretions by aspiration prior to autopsy. In this way, they became familiar with the appearance of cancer cells in smears.

As soon as the material was obtained from actual cases later on, it was sent to the laboratory, where smears were made immediately and, while still wet, were fixed in equal parts of 95 per cent alcohol and ether and stained by the Papanicolaou technic[12] (page 2174). When the amount of material obtained was small, all was removed from the collector and smeared, but when the amount was greater than could be utilized, it was first poured into a Petri dish, placed on a black background, and only those portions that resembled bits of sloughed tissue, or that were streaked with blood were smeared. All sputa secured from patients with positive bronchial smears were treated in the latter manner. At first thick smears were made, but it soon became apparent that thinner ones were more valuable, for in these all cells could be adequately studied. In preparing the smears the material was placed in the center of the slide and gently crushed with another slide, care being taken that any granules which may have been present did not escape peripherally. Every portion of the slide was systematically examined.

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[1] Herbut, P. A., and Clerf, L. H.: J. A. M. A. **130**: 1006, 1946.

[2] Sexton, D. L., Pike, G. M., and Nielson, A.: J. A. M. A. **116**: 700, 1941.

[3] Menninger, W. C.: J. A. M. A. **91**: 14, 1928.

[4] Poole, A. K.: Yale J. Biol. & Med. **1**: 345, 1929.

[5] Wile, U. J., and Benson, J. A.: Ann. Int. Med. **13**: 1243, 1940.

[6] Fishberg, Maurice: Arch. Int. Med. **37**: 745, 1926.

[7] Weller, C. V.: Arch. Path. **7**: 478, 1929.

[8] Edwards, A. T.: J. Thoracic Surg. **4**: 105, 1934.

[9] Craver, L. F.: Surgery **8**: 947, 1940.

[10] Holinger, P. H., Hara, H. J., and Hirsch, E. F.: Ann. Otol. Rhin. & Laryng. **54**: 1, 1945.

[11] Ochsner, A., Dixon, J. L., and Debakey, M.: Clinics **3**: 1187, 1944.

[12] Moss, R. E., and Long, W. E.: Arch. Dermat. & Syph. **46**: 386, 1942.

Pathology

Macroscopic.—In all cases reported by these authors in which cancer cells were found, the secretions were quite characteristic although not necessarily pathognomonic. The amount varied from a few drops to 4 c.c. The color ranged from gray and watery to somewhat creamy and opaque, but invariably it was either streaked with or diffusely mixed with bright red, pink, or dark brown partially disintegrated blood. It was always tenacious and adhered closely to the side of the collector and glass slide. It should be noted, of course, that blood-tinged secretions are not always indicative of cancer.

Microscopic.—One must mention the **noncancerous flora** of bronchial secretions. Most patients undergoing bronchoscopy have some pulmonary disease and therefore one can always expect to find leukocytic cells. The most commonly encountered and readily recognized are polymorphonuclear leukocytes (Fig. 650). They differ from those in blood films only in that their cytoplasm appears light green. Lymphocytes are small, round, and uniform, and contain a scant amount of light green cytoplasm. Plasma cells are present in varying numbers and show excentric nuclei and well-defined abundant light green staining cytoplasm. Mononuclear phagocytes are sometimes scanty but at other times are numerous. Their cytoplasm is always abundant, is sharply defined, stains light green, and frequently contains carbon or other phagocytosed material. Their nuclei are relatively small, round, evenly stained, and central or excentric in position.

Attention is called to the fact that every bronchial secretion contains sloughed epithelial cells which can be readily recognized as noncancerous. In most sputa one finds large, polygonal, sharply defined, pavement cells with relatively small round evenly stained nuclei. Occasionally a few of these cells are also found in nonneoplastic bronchial secretions, where they probably arrived by aspiration from the upper air passages. Ciliated columnar epithelial cells are practically always present (Fig. 650). They have a long slender shape with a pointed base, a central, round or oval, evenly stained nucleus, abundant light green cytoplasm, and a broad, sharply defined inner border often covered with numerous cilia. When these cells undergo degeneration their outlines break; the cytoplasm becomes irregular and frayed, and the nuclei become swollen, round, and faintly stained. Sometimes columnar cells appear in dense clusters and superficially resemble groups of stratified squamous epithelial cells. Single or small groups of cells, probably derived from the intermediary layers of the epithelium, are not uncommonly present and are important to recognize

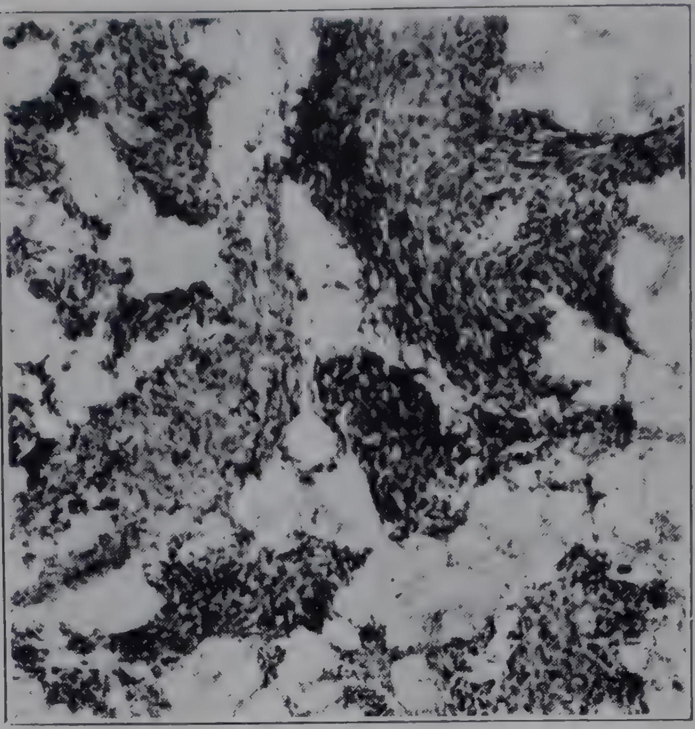

Fig. 640.

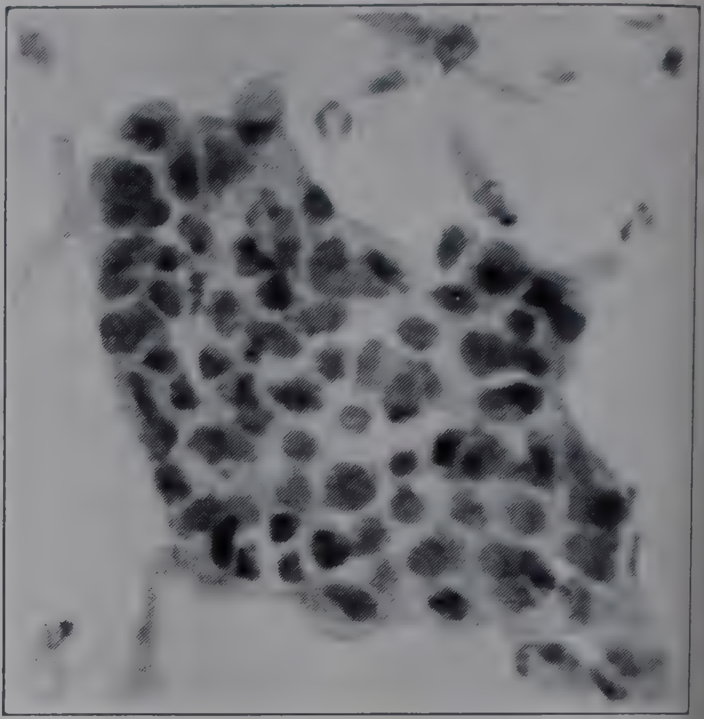

Fig. 641.

Fig. 640.—Smear of secretions removed endoscopically from a proved case of carcinoma showing four large pieces of tumor in squamous cell formation. The cells stain from pink to orange. Papanicolaou stain ×100. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Fig. 641.—A group of neoplastic cells in squamous formation removed endoscopically from another case of proved bronchogenic carcinoma. The cells are more compact and the nuclei are more irregular and relatively smaller than are those of noncancerous intermediary epithelium shown in Fig. 651. The cytoplasm of the cells illustrated in Fig. 641 stains pink to orange, whereas that in Fig. 651 stains a bluish green. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

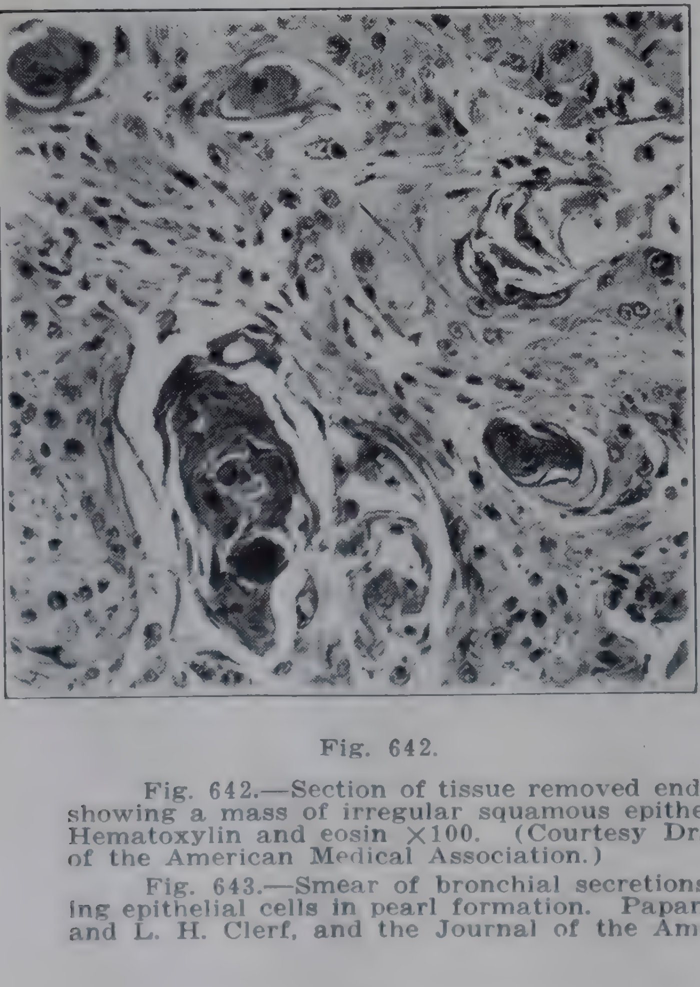

Fig. 642.

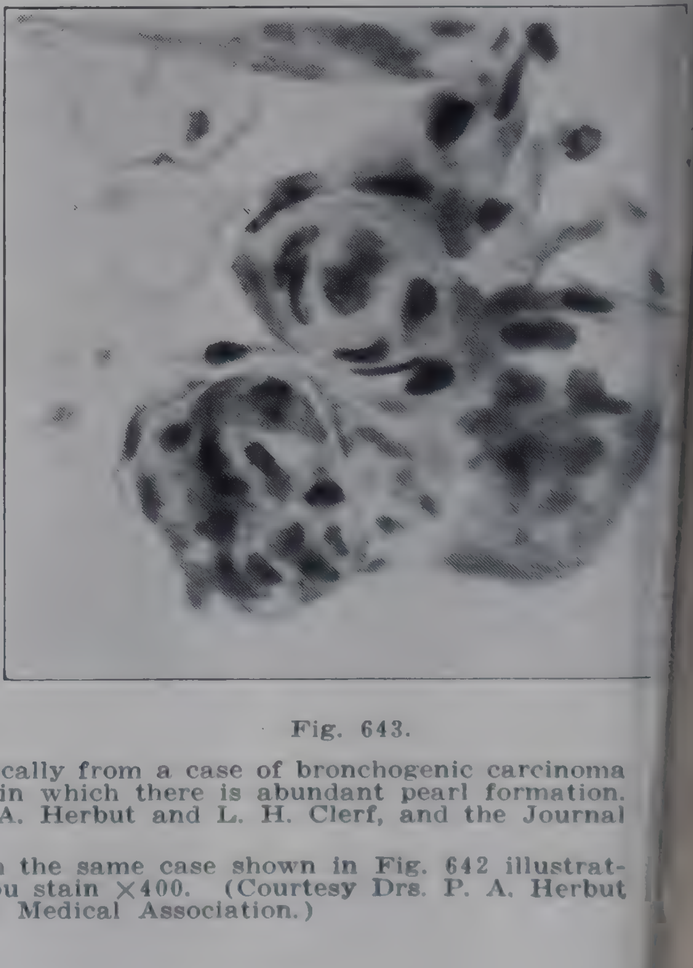

Fig. 643.

Fig. 642.—Section of tissue removed endoscopically from a case of bronchogenic carcinoma showing a mass of irregular squamous epithelium in which there is abundant pearl formation. Hematoxylin and eosin ×100. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Fig. 643.—Smear of bronchial secretions from the same case shown in Fig. 642 illustrating epithelial cells in pearl formation. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

as such (Fig. 651). They are of moderate size, round, oval, or polygonal, have sharply defined outlines, blue to green or gray cytoplasm, and relatively large, round or oval, evenly stained nuclei.

Cancerous Flora.—There is a certain appearance, difficult to describe precisely, but which gives one the impression that the preparation should contain cancer cells. In such slides leukocytic elements and fibrin are usually absent. This is particularly true of the macrophages. When these are numerous, cancer cells are not found. Pavement cells, most of which take an orange stain, are often increased in secretions containing cancer cells when the tumor is of a squamous cell variety. Finally, in cancerous secretion erythrocytes are usually fairly abundant.

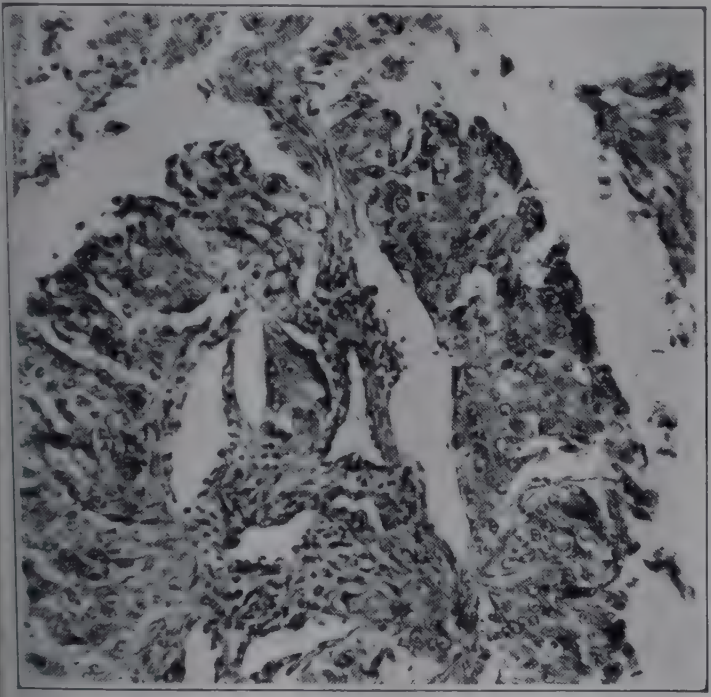

Fig. 644.

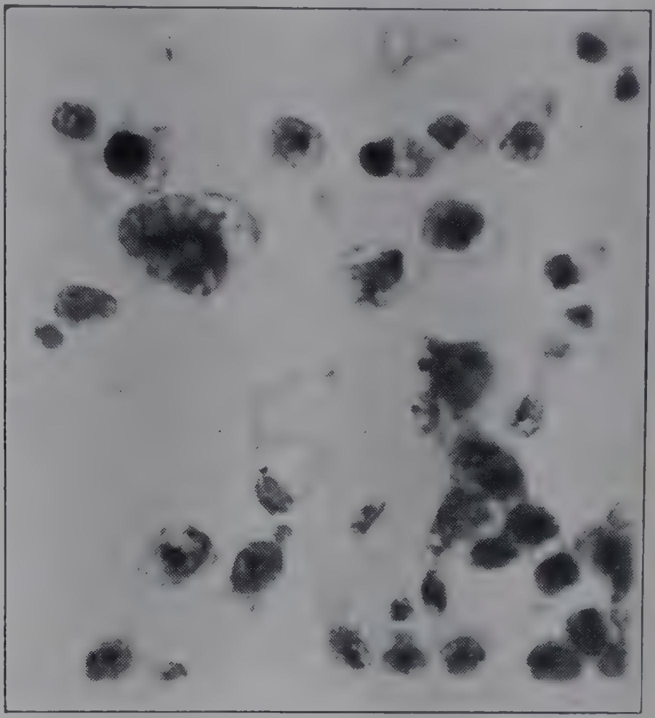

Fig. 645.

Fig. 644.—Section from a case of bronchogenic carcinoma obtained at necropsy showing groups of neoplastic cells partly differentiated into squamous cells but without keratinization or pearl formation. Hematoxylin and eosin ×100. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Fig. 645.—Smear of tumor from the same case illustrated in Fig. 644 showing a group of cancer cells of varying shapes and sizes. The nuclear membrane is quite distinct and the nucleoplasm is somewhat "washed out" and clumped. The cytoplasm is relatively scanty. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Cancer Cells.—These writers recognized three more or less separate groups of cancer cells in bronchial secretions: (1) clusters of cells which are present in squamous cell carcinoma; (2) large irregular single cells found in the more dedifferentiated tumors and in those with an adenomatous formation; and (3) smaller, more uniform cells present in the so-called "oat cell" carcinoma.

In squamous cell carcinoma there are usually small or large pieces of stratified squamous epithelium evenly distributed throughout all slides or present only in one portion of one slide (Figs. 640 and 641). If they happened to have been crushed at right angles to the surface one can see the keratinizing cells on the surface, the intermediate prickle cells, and even the basal cells. Usually, however, they are seen as a mass of closely packed, relatively large cells whose cytoplasm stains an orange red, and whose nuclei are large and dark blue. Cilia are never present. At the periphery there are usually encountered a few detached single cancer cells. They have an abundant amount of blue green to gray, ill-defined, peripherally frayed cytoplasm and relatively large, round, oval, or slightly irregular deeply stained nuclei. These cells are quite well differentiated and so do not appear as "wild" as do the cells of the more anaplastic tumors to be described presently. Occasionally when there is abundant pearl formation in the original tumor one finds naked pearls within the bronchial secretions (Figs. 642 and 643). They are similar to those seen in ordinary packed cells with deep staining, elongated, pyknotic nuclei, and bluish green to gray cytoplasm.

When the tumor is less well differentiated, in adenomatous formation or a combination of the two, single cancer cells are found in the secretions (Figs. 644, 645, 646, and 647). As would be expected they vary tremendously in size, shape, and staining qualities. They are invariably considerably larger than normal cells (sometimes of giant proportions) and assume anything but the regular configurations described. Their borders are almost always ill defined, frayed and fuzzy. The cytoplasm stains bluish green, blue, or gray, and although absolutely increased it is relatively decreased and sometimes almost entirely imperceptible. The nuclear-cytoplasmic ratio is thus always increased over the normal. While theoretically some of the cells should be vacuolated, this appearance has not yet been encountered. The nuclei are single or multiple and usually are extremely bizarre. Whereas they may be round or oval they ordinarily form irregular, coiled, folded, hexagonal or dumbbell shaped configurations. Some of the larger and more regular appearing nuclei show a very sharp, deeply stained, peripheral membrane which enclose lightly stained somewhat "washed out" and clumped nucleoplasm. Other nuclei are solid and

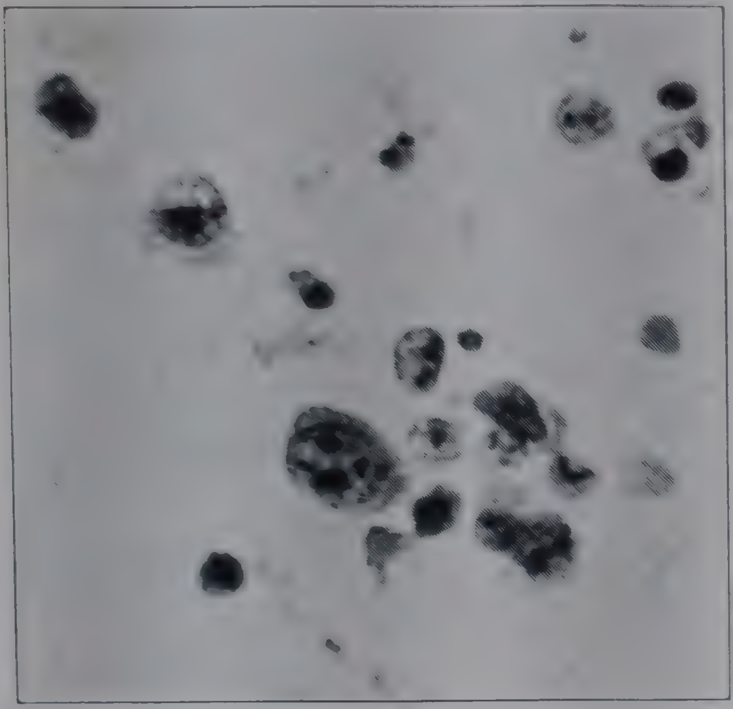
Fig. 646.

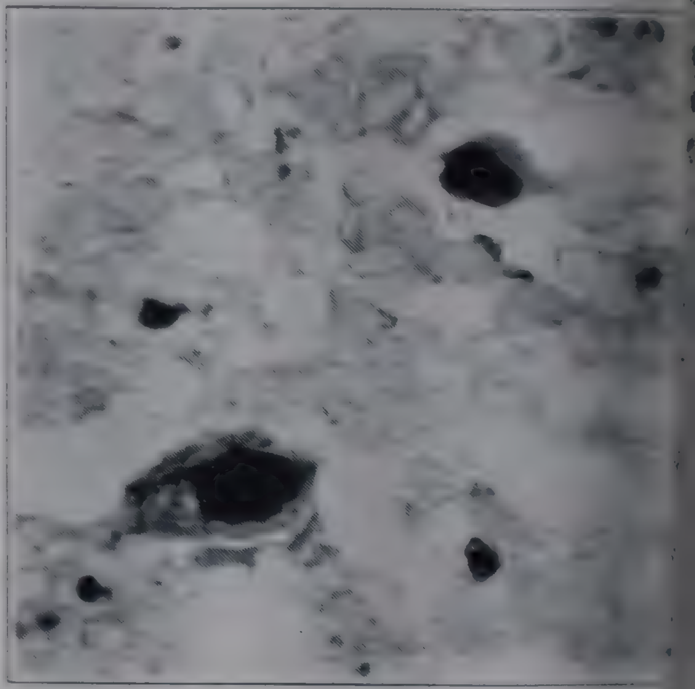
Fig. 647.

Fig. 646.—Smear of bronchial secretions from the same case illustrated in Fig. 644 showing tumor cells identical with those seen in Fig. 645. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Fig. 647.—Smear of secretions obtained endoscopically from a case of proved moderately well-differentiated bronchogenic carcinoma showing two cancer cells. The cytoplasm stains a foggy bluish gray, the nuclear-cytoplasmic ratio is increased, and the nuclei are extremely hyperchromatic. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association).

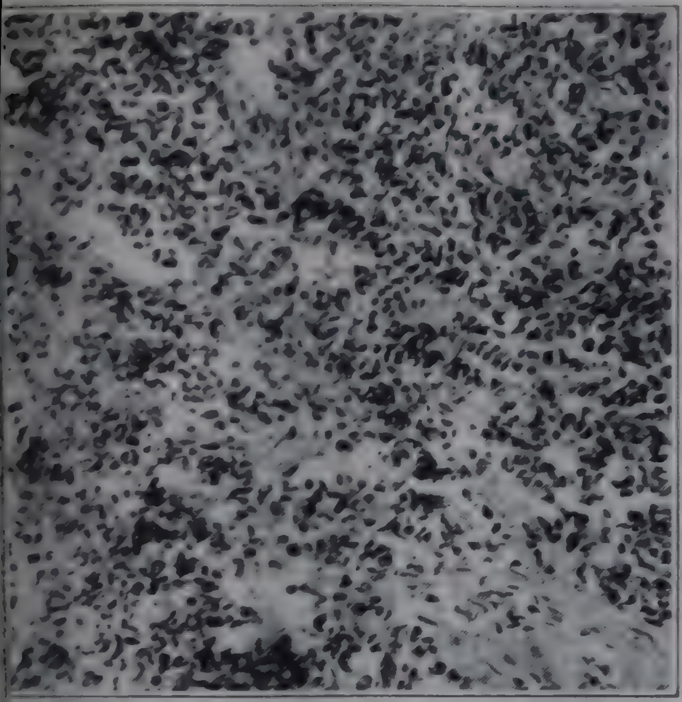

Fig. 648.

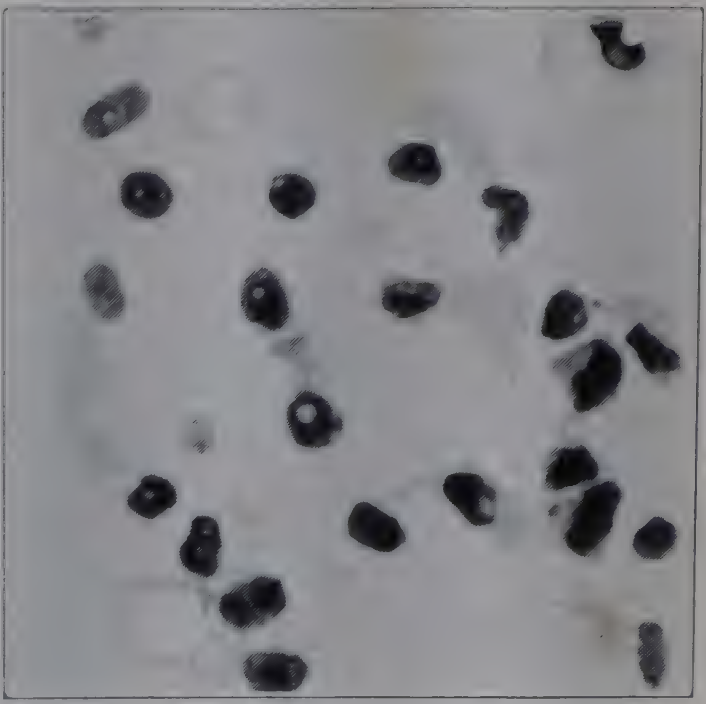

Fig. 649.

Fig. 648.—Section of tissue from a case of bronchogenic carcinoma of an "oat cell" variety obtained at necropsy. Hematoxylin and eosin ×100. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

Fig. 649.—Smear of tumor from the same case illustrated in Fig. 648 showing numerous anaplastic cancer cells. They contain practically no cytoplasm and deeply stained irregularly shaped nuclei. Note the presence of one or more nucleoli within a single nucleus. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

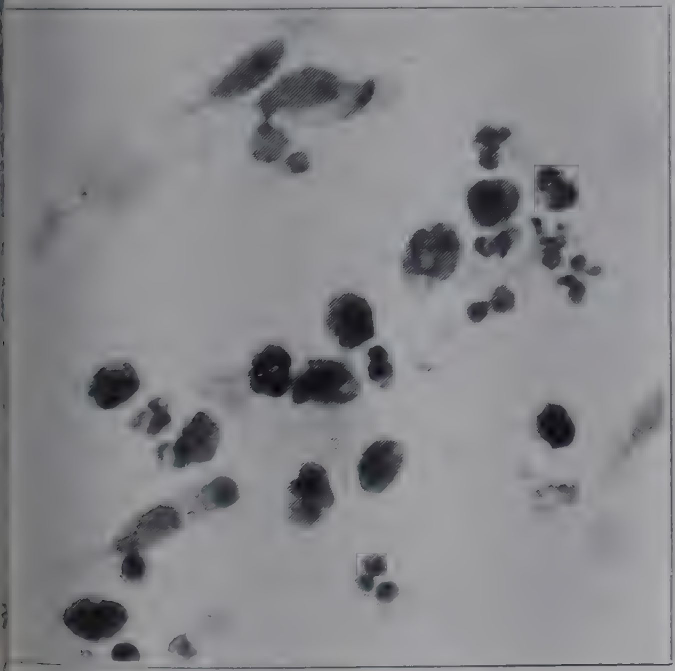

Fig. 650.

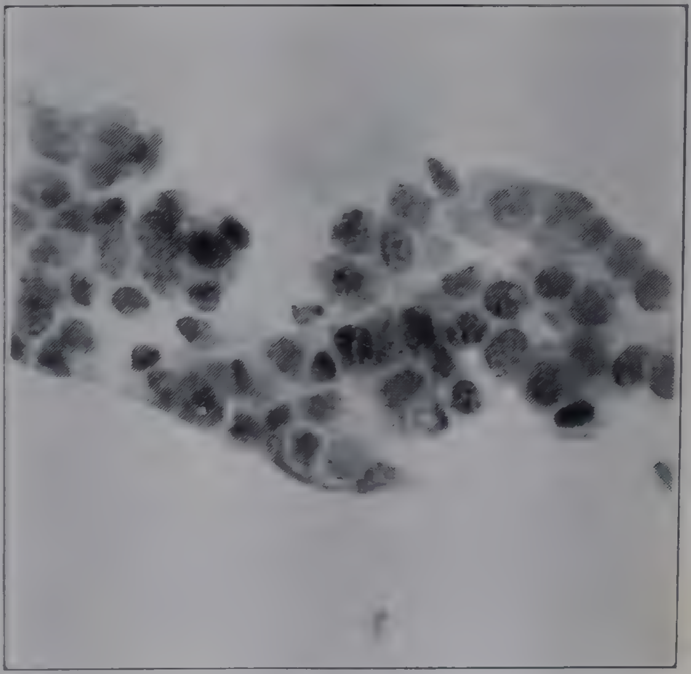

Fig. 651.

Fig. 650.—Smear of bronchial secretions obtained from the same case illustrated in Fig. 648. There are ten cancer cells identical with those shown in Fig. 649. There are present also a few polymorphonuclear leukocytes, one normal columnar epithelial cell with cilia, and two partly degenerated columnar epithelial cells in the lower portion of the photomicrograph. Papanicolaou stain ×400. (Courtesy of Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.

Fig. 651.—Smear of bronchial secretions removed endoscopically from a case of pulmonary tuberculosis showing a group of noncancerous intermediary cells as compared with cancer cells seen in Fig. 649. They are loosely united and contain large evenly stained nuclei and a relatively small amount of cytoplasm that stains bluish green. Papanicolaou stain ×400. (Courtesy Drs. P. A. Herbut and L. H. Clerf, and the Journal of the American Medical Association.)

so hyperchromatic that the internal structure is not discernible. Nucleoli are not always demonstrable but, when present, they are easily recognizable as round or slightly oval, sharply circumscribed bodies stained either lighter or heavier than the surrounding nucleoplasm. Often as many as four can be seen within a single nucleus.

As compared with the foregoing cells, neoplastic cells derived from the so-called "oat cell" carcinoma are relatively small (Figs. 648, 649, and 650). They are round, oval, or slightly irregular, contain a scanty amount of blue to green cytoplasm, and possess round, oval, or somewhat irregular, sharply defined, deeply stained single nuclei. They are best identified by their size and by the fact that although the cells are similar there are definite variations in the shape of the nuclei from cell to cell.

In 30 consecutive cases of primary pulmonary carcinoma, bronchial secretions were stained by the Papanicolaou technic and studied. Cancer cells were demonstrated in 22 cases, or 73 per cent. Cancer cells were present in the secretions from 7 cases in which bronchoscopic findings were negative. In only 1 of 5 cases in which cancer cells were present were neoplastic cells found. This is not a substitute for any other method of making a diagnosis of bronchogenic carcinoma but it is an adjunct to the means already employed. It is particularly useful in cases in which the tumor is located at the periphery of the lung or in the upper lobe.

BIOPSY TECHNICS FOR INTERTHORACIC CONDITIONS

In addition to bronchoscopic and esophagoscopic biopsies, bronchial washings, cytologic examinations of sputum for pulmonary carcinoma, there is also recommended the following: (1) resection of the deep cervical fat pad and nodes; (2) mediastinal biopsy at open thoracotomy; and (3) lung biopsy through an open thoracotomy. Resection of deep cervical fat pad and continued nodes is the simplest of the three procedures, and is the best withstood by even poor risk patients. It can be performed under local anesthesia.

CYTOLOGIC DIAGNOSIS OF GASTRIC MALIGNANCY

Ever since the advent of the Papanicolaou cytologic methods, the diagnosis of gastric cancer has been hampered because cancer cells are acted upon adversely by the gastric juice and so altered that diagnosis is difficult if not impossible in most cases. Early reviews on this subject show that about one-third of the cases of gastric cancer were diagnosed by routine aspirated specimens. Tomenius[1] reported an elaborate procedure for securing better preservation of gastric cells. Papanicolaou and Cooper,[2] Hollander, Hess, and Sober,[3] Ulfelder, Graham, and Meigs,[4] Richardson, Queen, and Bishop,[5] and Botsford and Tucker[6] have reported various technics for improving the collection of gastric aspirates for diagnosis of cancer. All reports show that the clinical application has been limited by frequently missed diagnoses.

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[1] Am. J. Digest Dis. 16: 425, 1949.
[2] J. Nat. Cancer Inst. 7: 357, 1947.
[3] J. Nat. Cancer Inst. 7: 365, 1947.
[4] Ann. Surg. 128: 422, 1948.
[5] Am. J. Clin. Path. 19: 328, 1949.
[6] J. A. M. A. 142: 975, 1950.

Seybolt, Papanicolaou, and Cooper[7] revealed that about one-third of the cases of gastric cancer were diagnosed by the routine aspirated specimen and that these results could be improved by means of careful gastric lavage. It was thought that mechanical irritation of the gastric mucosa might produce better diagnostic material. For this purpose an inflatable abrasive balloon has been devised and tested.

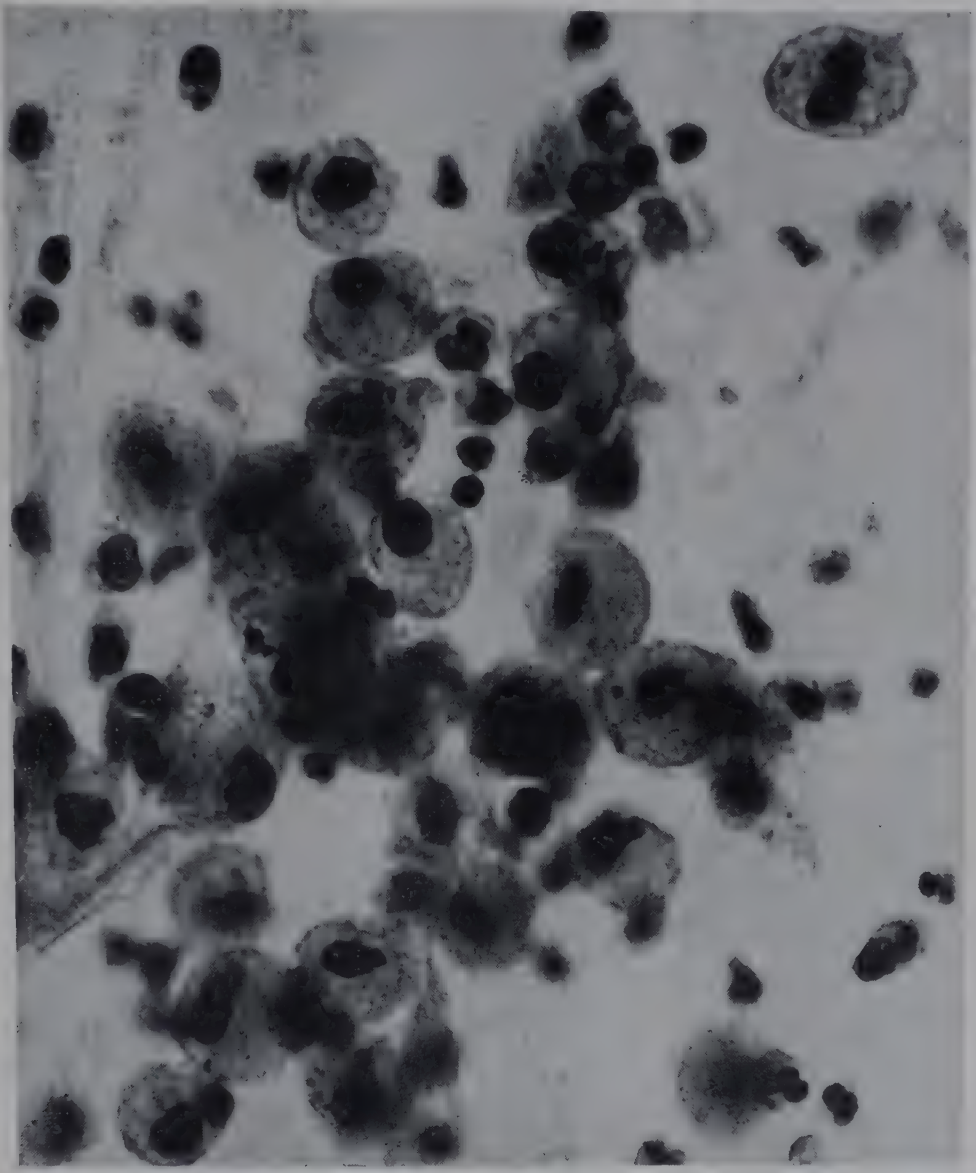

Fig. 652.—Bronchial washings showing large cancer cells. (×680.) Case 156632, Gradwohl collections.

Panico, Papanicolaou, and Cooper[8] described an apparatus for obtaining gastric secretions after mechanical irritation of the mucosa. A later paper by Cooper and Papanicolaou[9] reported further results with this method. With the two papers just cited, abrasive balloon technic has been described with the results which are tabulated. During the first year of trial with the balloon device, the studies were confined to patients in whom the pathology was known. This procedure was carried out in these known cases to learn at the outset which portion of the material obtained by using the balloon was likeliest to obtain well-preserved groups of cells. It was necessary to learn which was the best material, that aspirated while the balloon was in the stomach, that on the balloon after it was removed, or that which remained in the stomach after the balloon was withdrawn. Many extensive comparative studies were made in each patient. It was found that the best cell groups were found on the balloon itself. Material on the balloon often showed gross particles of tissue which could be smeared separately. The remaining material was washed off the balloon in Ringer's solution.

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[7] Cytology in the Diagnosis of Gastric Cancer, Cancer **4**: 286, 1951.
[8] J. A. M. A. **143**: 1308, 1950.
[9] J. A. M. A. **151**: 10, 1953.

The apparatus (Fig. 653) for the test consists of a standard 16 French double-lumen tube 100 cm. long, with markings at 45, 60, and 75 cm. One lumen, designated the *aspirator,* is connected proximally to a syringe and distally to a patent, silver-plated bucket. The other lumen, designated the *agitator,* is connected proximally to an inflating bulb and distally to an inflatable balloon. The balloon is made of a condom open at both ends. To the external surface are tied by single slipped bowknots approximately 250 pieces of untreated braided silk, arranged in a regular pattern about 3 mm. apart and cut to leave ends about 2 mm. long. Ends of the balloon are fixed to the tube by silk ties about 8 cm. apart. When the balloon is uninflated it collapses on the sides of the tube; when it is inflated with 175 c.c. of air it measures 10 by 5 cm.*

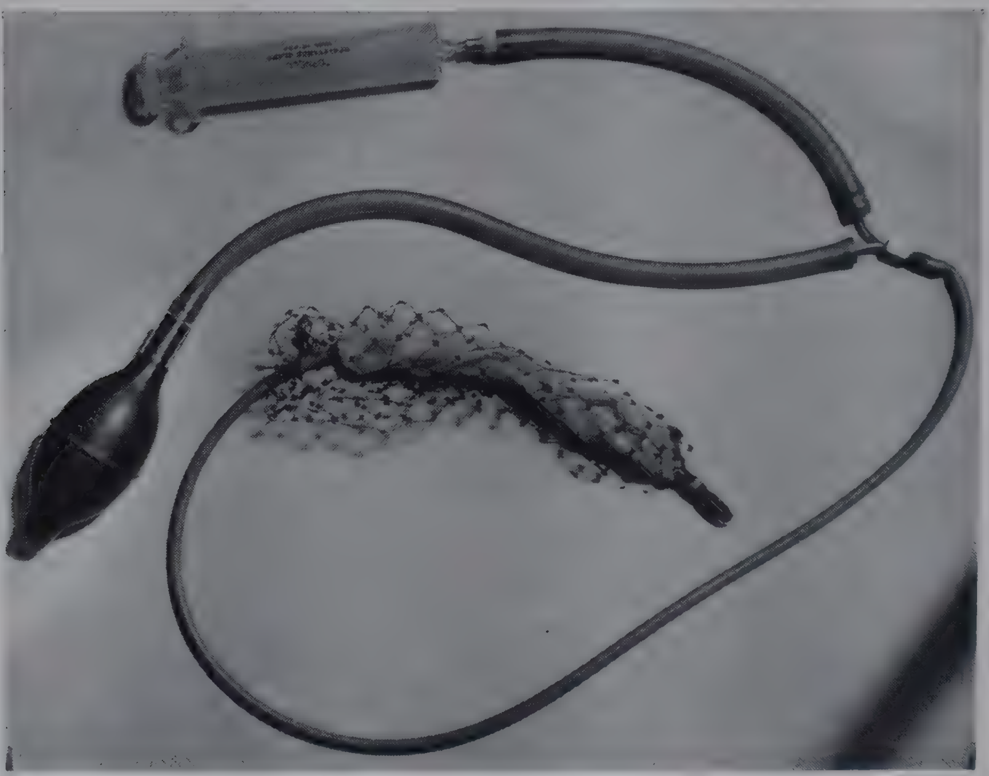

Fig. 653.—The inflatable gastric balloon apparatus of William A. Cooper, Frederick G. Panico, and George N. Papanicolaou.

The test is made in the morning, all food and fluid being withheld for the previous eight hours. If the patient has gastric retention or has had barium the day before, this may not be adequate preparation. If the stomach is found to have retained food particles, any quantity of fluid, or barium, the test should be abandoned for that day and the stomach further prepared. A day of liquid diet followed by a thorough lavage the night before will be adequate in all cases except those with marked gastric retention. In such cases, several days of a liquid diet followed by nightly lavages or continuous aspiration the night before may be necessary to clear the stomach of all retained debris. This is emphasized because the most essential factor in obtaining good specimens is a clean dry stomach.

All dentures are removed, the patient's mouth is rinsed, and the patient is instructed to expectorate all saliva. The deflated balloon is wet in Ringer's solution and passed orally, with small sips of the same solution taken through a drinking tube. Most patients swallow the balloon easily. If any great difficulty is encountered, the gag reflex may be suppressed by a little 2% solution of tetracaine (Pontocaine) hydrochloride spray.

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*The entire apparatus can be purchased from Dr. Arthur O. Dahlberg, Walbrook Road, Scarsdale, N. Y. Full directions for using are given with the instrument.

When the tube is past the 60 cm. mark, the balloon is in the stomach.

The stomach is then aspirated until dry, and this material is saved as the fasting specimen for a gastric analysis. Histamine is then given, and three 10-minute specimens are collected for gastric analysis, using the continuous aspiration technic. After 30 minutes of aspiration, the stomach is clean and dry. The balloon is then inflated with about 150 c.c. of air, and the tube is withdrawn until the balloon is checked at the cardia. When at the cardia, the inflated balloon will not come up any further and the patient may gag. Peristaltic action then carries the balloon down to the pylorus in 5 to 10 minutes. The balloon is deflated, reinflated, and pulled back to the cardia while inflated. This process is repeated five or six times over a period of 30 minutes. While the balloon is down, the stomach is kept dry by frequent aspiration. This material is usually discarded, but it can be saved for processing in cases where the examination is being repeated, for it often contains well-preserved groups of cells. It may also be saved and processed if the material on the balloon is scanty.

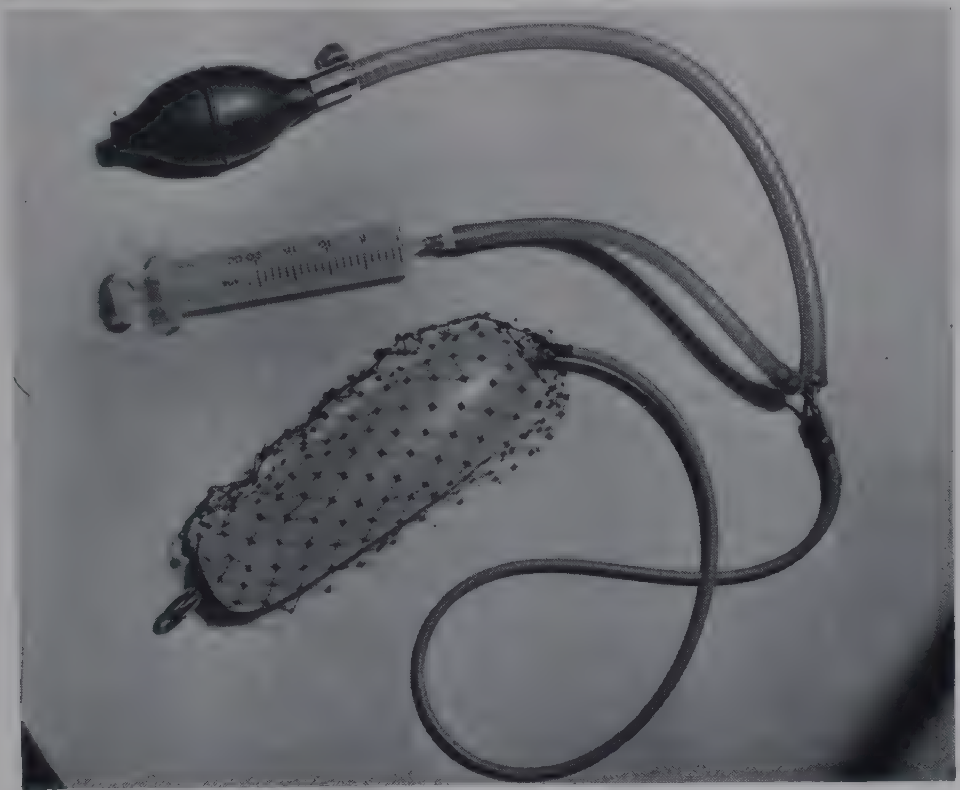

Fig. 654.—The inflatable gastric balloon apparatus, showing the balloon partially inflated.

The number of smears should be kept to a maximum of five and still there should be on these slides as much good material and as little debris as possible. If the aspirated material is saved, it should be put in 95% alcohol as it is collected.

Material when aspirated is often pink, and might contain blood. It is likely to contain fresh blood if a gastric lesion is present. If the amount of blood is in any way alarming, the test should be terminated. There have been no gastric hemorrhages or untoward reactions in over 300 clinical trials with the balloon method, although some fresh blood has been obtained in a number of cases.

The time of 30 minutes to leave the inflated balloon in the stomach has been selected arbitrarily; in some cases the time has been reduced and specimens from these patients have been perfectly adequate. In a recent case reported by Cooper, a friable papillary carcinoma yielded an excellent specimen in 10 minutes.

When the balloon has been down an hour, it is deflated and withdrawn. The balloon is then inflated again and inspected for gross particles. These particles are smeared on slides that have been prepared with albumin. In this way, the largest fragments of tumor tissue are likely to be obtained in their purest form; some of the best specimens have been obtained in this way.

The balloon is then rinsed vigorously in about 200 c.c. of a mixture of equal parts of Ringer's solution and 80% alcohol. This puts into suspension the particles clinging to the net. The solution is then centrifuged for 30 minutes at 1,500 r.p.m. and the sediment smeared on two to four slides, depending on the quantity of sediment.

Smears are then fixed and stained according to the Papanicolaou technic. Once the smears are fixed, delay is not important, but the material should not be allowed to stand around for more than an hour at any time before fixation.

The stained smears are read as class 1 or 2, which are considered negative; class 3, which is suspicious; and class 4 or 5, which are considered positive. All tests reported by these workers were done before operation.

In the last communication by Cooper and Papanicolaou, there were 7 frank errors in the 238 diagnostic problems, an over-all accuracy of 97.1 per cent. In this same series of cases, the accuracy of the x-ray examination was about 85 per cent. Therefore, this technic offers a diagnostic method with an accuracy that far exceeds that of any other method available at the present time.

Consideration must be given to this procedure as an aid in the early diagnosis of gastric cancer. As a matter of fact, about two-thirds of the patients seen are inoperable and really small gastric cancers are a rarity. But it must be remembered that three of the cancers in these series were lesions of less than 1 cm. in diameter and two had strongly positive smears. The only small lesion that was missed cytologically was a carcinoma in situ 2 mm. in diameter, which was discovered only by serial section of what appeared to be a benign ulcer. This evidence suggests that the method may be accurate in the detection of early gastric cancer. An absolute estimate will have to wait the application of the balloon smear technic to groups of patients in the presymptomatic, or at least early symptomatic, phases of the disease. This proof will not be come by easily or quickly, but as the method becomes more widely known and generally used, it is felt that some cases of gastric cancer will be discovered in their earlier phases.

As Cooper and Papanicolaou aptly state, "Earlier diagnosis cannot help but result in more cures of this disease."

Gastric Lavage With Papain-Cysteine Solution for the Diagnosis of Gastric Carcinoma

Attention has been drawn by Rosenthal and Traut[1] to the mucolytic action of papain for cell concentration in the diagnosis of gastric malignancy. They suggested the use of a buffer of approximately pH 7.3 for the purpose of neutralizing excess acid. Actually, papain is active over a wide pH range. The buffer used is that used in Ringer solution.

Buffer.—

Sodium dibasic phosphate ($Na_2HPO_4.2H_2O$)	350 gm.
(If the anhydrous form or the form with 12 molecules of water of crystallization is used, adjust the weight accordingly.)	
N/1 hydrochloric acid	400 c.c.
Distilled water	5 gallons

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[1] Cancer 4: 1, 1951.

Papain-Cysteine Solution.—

Grind 3 level teaspoonfuls of papain in a mortar with ¾ teaspoon of cysteine hydrochloride (activator).

Stir briefly in the buffer solution and filter through a coarse filter paper. The ordinary fine filter paper becomes clogged.

Make fresh, every day.

Technic.—

Discard the fasting specimen.

Introduce one pint of papain solution through an Asepto syringe, the mouth of which is introduced into the end of an ordinary flared Levine tube.

After 10 minutes, remove from the stomach as much of the solution as can be obtained. Usually most of the solution is recovered. Rarely all is lost because of duodenal passage. This has not caused any apparent harm.

Centrifuge the lavage fluid if a large centrifuge is available; or if not available, take an aliquot after mixing.

Continue centrifugalization at moderate speeds for 5 to 10 minutes.

Discard the supernatant fluid.

Add to the sediment a drop of Gastric Mucin, Armour, and stir thoroughly. This restores adhesiveness to the cells. An alternate method is to add a pinch of Mucin to the digest just before centrifugalization.

Place a large drop of the sediment on a slide.

Spread by pressing with another slide in the same manner that cover slip blood spreads are made.

Gently pull the two slides apart and place them immediately into the fixative of equal parts of ether and 95% alcohol.

Carry out fixation and staining in the usual manner, using the Papanicolaou method.

The material should be processed preferably within one-half hour to avoid digestion of the cells.

There is a theoretical risk of provoking a hemorrhage by digestion of fibrin in the base of a gastric ulcer. Papain has a proteolytic action similar to that of pepsin. However, no undue hemorrhage has taken place in 400 examinations (Rosenthal and Traut), including approximately 40 gastric ulcers. Microscopically, blood is often seen, however. Three patients with previous massive hemorrhages (approximately two weeks previously) have been examined without provocation of hemorrhage.

CYTOLOGIC DIAGNOSIS IN UROLOGY

Foot, in his classic work, *Pathology in Surgery,** classifies tumors of the kidney as (1) papillary adenocarcinoma; (2) clear-celled carcinoma or renal cell carcinoma; (3) papillary carcinoma with granular cells; (4) malignant adenoma; (5) tubal adenocarcinoma; and finally (6) hypernephroma. Tumors of the urinary bladder are classified as papilloma, which is a transitional-celled papilloma, or an "overgrowth of the lining epithelium that takes the form of a patch of villous projections that float in the urine like clusters of seaweed"; epidermoid papilloma; carcinoma; transitional-celled carcinoma; keratinizing carcinoma; diffuse carcinoma; aniline tumors, apparently caused by long exposure to alpha- and beta-naphthylamin and benzidine; and epidermoid carcinoma.

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*J. B. Lippincott Company, 1945.

Tumors of the prostate, as classified by Foot, are adenoma, leiomyoma, carcinoma, adenocarcinoma, of which there are several types, but they can be grouped under two headings, multiacinar form and large acinar form; myosarcoma and rhabdomyosarcoma.

The question of whether or not exfoliative cytologic methods will be helpful in the diagnosis of malignancy of the urogenital tract was taken up in a special course given by Papanicolaou and the clinical staff at Cornell University Medical School in New York in 1948. In this course, Marshall, of the Cornell University Medical School, stated that malignant cells are easily found in prostatic cases; also that this method is not useful in all clinical cases because at times biopsy of the tumor may be necessary. Papanicolaou reported on the advisability of using his method of exfoliative cytologic examination in suspected carcinoma of the prostate. Gradwohl and Hall,[1] 1948, reported their results on examination of numerous cases of known and unknown carcinoma of the prostate. These examinations were carried out by testing the urinary sediment by the Papanicolaou method. They corroborated the statements of Cahill,[2] that cytologic examination of the urine is of importance in these cases, as has been stressed by Papanicolaou. This may be of the gross urine, or may be material of ureteral specimens. The examination in which the sediment is fixed and mounted as tissue requires experience as to interpretation because of the changes occurring in the cells due to their immersion in the urine. In parenchymatous tumors, two types of cells are seen: (1) unusually large cells with mitotic changes, and (2) vacuolated cells with clear cytoplasm, rich in lipids, so characteristic of clear-celled carcinoma or hypernephroma. Cytologic studies in all urinary tumors is an essential step in the diagnosis. It is our belief that cytologic study of the urine will prove of great value in the diagnosis of renal neoplasm and for the diagnosis of cancer of the urinary tract.

Technic.—

The method used is essentially that suggested by Papanicolaou.

Obtain a specimen of urine, preferably catheterized, and mix with equal parts of 95% alcohol.

Centrifuge as soon as possible, at medium speed, for 30 minutes.

Remove the supernatant fluid.

If smears cannot be made immediately, cover the sediment with absolute alcohol, and place in a refrigerator.

To make smears, prepare a light film of Mayer's albumin fixative on slides and cover with a small amount of sediment and then spread with another slide as evenly as possible.

When it is noted that the edges are drying, while the center is still moist, fix the slide in equal parts of alcohol and ether for a minimum of one hour.

Stain exactly as described for vaginal smears (page 2174).

For the diagnosis of prostatic cancer, the recommended method is as follows: Have the patient void part of his urine, then massage the prostate. After this, have the patient void the remainder of the urine, which will contain prostatic massage fluid. Examine both specimens as indicated above, by centrifugalization and smearing the sediment over a thin film of Mayer's albumin fixative. When the film begins to dry around the edge, fix it in equal parts of alcohol and ether, and stain in the usual manner.

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[1] Urol. & Cutan. Rev. 53: 237, 1949 (reported October, 1948).

[2] J. A. M. A. 138: 5, 1948.

SOLUTIONS FOR THE PRESERVATION OF MUSEUM SPECIMENS

Kaiserling Method

The Kaiserling method gives by far the best results for ordinary pathologic and anatomic preparation where the color of the red blood cells is to be preserved. If any of the faint yellows or greens (due to bile staining or other causes) are to be kept, one of the chloral hydrate fluids, preferably Klotz, gives excellent results, with Kaiserling No. III as a permanent preservative.

Specimens must be obtained fresh from the autopsy or operation and not be allowed to soak in water for more than a few moments. Only the superfluous blood should be washed away, remembering that the one color available for preservation lies in the hemoglobin of the red blood corpuscle, and that this, therefore, must be preserved. The basis of the methods of color preservation which are to be recommended lies in the chemical change produced by the action of formaldehyde upon the hemoglobin of the red cell and its subsequent fixation as the red alkaline hematin by alcohol.

It is necessary to enlarge on the proper laying out of the specimens in the No. I solution. No cloth, metal pins, nor paper must be allowed to come in contact with them. Cotton wool to hold in shape and stretching on glass frames with cotton thread is all that is necessary. Large bodies of fresh fluids must be used. After careful comparison both abroad and at home, the following process has been found to give the best results:

Kaiserling Solution No. I.—

Potassium acetate	170 grams
Potassium nitrate	90 grams
40% Formalin[1]	1,600 c.c.
Water	8,000 c.c.

Care should be taken to use only high grade chemicals.

1. **Water white formaldehyde** must be as free from formic acid as possible. It is not necessary to buy the chemically pure grade. A white deposit, due to freezing (paraformaldehyde) is sometimes seen in the Formalin. This lessens the concentration of formaldehyde gas, and consequently the color and fixing processes are not correct. Always buy Formalin in the spring of the year and see that there is no white deposit present. Do not boil it unless you restore the amount of Formalin used in making the solution.

If the formaldehyde is found to be acid, it can be neutralized with sodium carbonate or sodium hydroxide.

To make a 2% Formalin solution:

Formalin	5 parts
Water	95 parts

4%: Use 10 parts of Formalin to 90 parts of water.
6%: Use 15 parts of Formalin to 85 parts of water.

2. **Potassium acetate.** We recommend Mallinckrodt potassium acetate.

3. **Potassium nitrate.** Merck's pure powder, U.S.P., is excellent. (It is not necessary to use c. p.)

4. **Glycerine**, water white, c. p. Do not use dynamite glycerine as it produces a change in color.

5. **Chloral hydrate.**

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[1] Water white formaldehyde.

The length of time the specimen should remain in Kaiserling's first fixation fluid has caused a great deal of comment. It is of secondary importance to thorough hardening. The specimen should be fixed in such way that no blood will ooze out when firmly pressed between the fingers. In fact, good color results have been obtained in specimens that have stood from two to four months in the No. 1 solution. These are, of course, exceptional cases. Intestines should not be left for more than ten to twelve hours. Other specimens or organs should be left in the solution from one to five days. In all instances they must be thoroughly fixed. Formalin increases the firmness of tissue. The tissue loses its natural color and assumes an aged and grayish appearance.

Great care must be taken to wash the specimens thoroughly in running water after removal from this solution.

Kaiserling Solution No. II.—

Ninety-five per cent alcohol is used. It may be used at once without the intermediate stage of 80% alcohol recommended by Kaiserling. The specimens must be left here until the natural colors, which have disappeared under the action of the Formalin in the first solution, return, the process being watched in the same way as in the development of a photographic plate.

The strength of the alcohol should not be allowed to go below 80%. To avoid this the specimens should be transferred to a second alcohol of the original strength. The time in which the specimens remain in the alcohol varies according to the particular character of the tissue.

In this stage again the specimens must be carefully washed in water, no traces of the Formalin or alcohol used in the first and second solutions are to be carried to the final fluid. If either the alcohol or the Kaiserling or the Kaiserling No. III solution used gives off any odor of either Formalin or alcohol, it must be thrown away. This is absolutely necessary.

When specimens have been left in alcohol for too long a time, color can be restored by leaving them in equal parts of Kaiserling No. III and glycerine for twenty-four hours, allowing for the amount of glycerine already in the Kaiserling fluid.

Kaiserling Solution No. III.—

Potassium acetate	4 lb.
Glycerine	2,000 to 4,000 c.c.
Distilled water	10,000 c.c.

to which 20 c.c. carbolic acid may be added as a preservative.

In preserving veterinary or comparative anatomy specimens Kaiserling is not satisfactory: the best results have been obtained by Klotz, increasing the Formalin possibly to 6%, then placing in the alcohol, and preserving in Kaiserling III.

When specimens in Kaiserling III in open jars are used to pass around a class, care must be taken not to allow the surface to dry, or the fluid to be contaminated with molds. One infected jar may cause contaminations in many others and necessitate putting them through No. I and No. II solutions just as you would with a fresh specimen.

To Clarify Kaiserling Solutions

To get a clear Kaiserling No. III, connect a five-gallon vessel with an air compressor. Run the Kaiserling through a Berkefeld filter. This takes twenty to thirty minutes. Run water through the filter to clean it. Be sure to connect the filter with the water main.

Another method of clarifying Kaiserling No. III follows:

For 10,000 c.c. of Kaiserling No. III, 5 funnels are required. Prepare filter paper. Do not fold it. Place the five filters in a row. Use one tablespoonful of charcoal (very fine bone charcoal) for each funnelful. Put the charcoal in a jar and shake well with Kaiserling No. III. Run the solution through the funnels slowly. Refilter the first filtrate that comes through the funnels.

In making Kaiserling No. III, do not use tap water. Use distilled water; tap water contaminates the solution.

Kaiserling No. III dissolves plaster of Paris.

Klotz formerly used a little formaldehyde in Kaiserling No. III. This is not recommended as it causes an undesirable chemical reaction. He has now changed to carbolic acid. Carbolic acid makes a specimen slightly gray but prevents the growth of fungi.

Important points to remember are: There must be no odor of formaldehyde or alcohol in Kaiserling No. III. Wash out both of these materials thoroughly so that no trace of odor remains. Overharden in Kaiserling No. I rather than underharden. Always sacrifice color to permanency. Avoid infecting the jars. If infected, specimen must be treated again with Kaiserling Nos. I, II and III.

Formalin will not penetrate either liver or lungs. In order to fix and preserve color, they must either be cut into slabs or injected.

For red blood cell color preservation, place the specimen in a Novy jar and cover with Kaiserling No. I. Then saturate with carbon monoxide gas from any gas jet. To eliminate excess gas, burn off the exhaust or connect a tube to the Novy jar and extend it out of the window.

Always protect a specimen from the direct rays of the sun even after it is completed. Colors are affected by sunlight. Diffuse sunlight will not affect them. Heat will destroy color. Keep specimens in a cool place.

Never preserve a specimen in pure glycerine. A spleen preserved in pure glycerine will shrink to one-half its size.

Be careful of the concentration of alcohol in Kaiserling No. II because it will lose its strength. Keep above 80%. Use a small alcoholometer employing a small test tube (Arthur H. Thomas Company).

Frost's Sugar Solution.—

Sodium fluoride	80 gm.
Chloral hydrate	80 gm.
Potassium acetate	160 gm.
Cane sugar (Tate's cubes)	3,500 gm.
Saturated thymol water	8,000 c.c.

Sodium fluoride may be omitted. Tate's cube is not necessary. Use cane sugar. Never, under any circumstances, use beet sugar.

THE HAIR IN POLICE TECHNIC[1]

In an extensive paper presented before the Academy of Science of Havana on "The Hair of the Races in Cuba," Dr. Israel Castellaños, Director-General of the National Bureau of Identification, Havana, Cuba, pointed out that valuable researches in comparative microscopy had been made public, but very few contributions treating of the hair in police technic had been made. The most important work in this field was done in Europe.

There is an abundant bibliography of anthropologic and medicolegal studies of the hair, but there are only short references to its value in police work. This may perhaps be accounted for on the basis of Gross's exclamation, in 1904: "How seldom clothes are examined, with a view to finding some hair! And if it is found, by mere chance, how seldom is it submitted to examination by an expert!"

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[1] Much of the material on hair in police technic and identification through the hair by both microscopic and chemical methods is abstracted by permission from the book, *Identification Problems—Criminal and Civil*, by Israel Castellaños, M.D., Director-General, National Bureau of Identification, Havana, Cuba, published by R. V. Basuino, Brooklyn, N. Y., 1939.

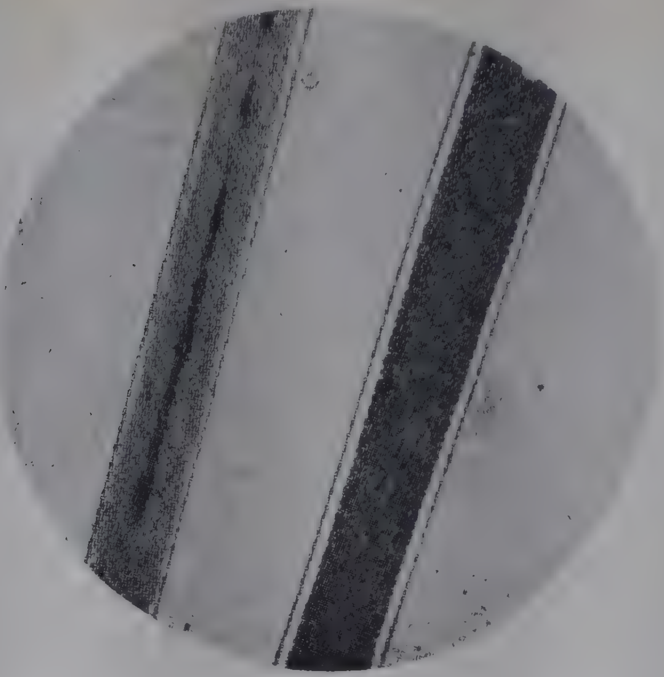

Fig. 655.—Human hair (left) and animal hair (right). These are differentiated by their three constituents—the cuticula, the cortex, and the medulla. In man, the cuticula is thin, slighted serrated, and imbricated. In animals, it is just the opposite. In man, the cortex shows small homogeneous pigment. In animals, the pigment is irregular and larger than in man. In man, the medullary channel is narrow, while in animals, it is much better developed, to such a degree that the greater part of the hair consists of the medulla. (Courtesy Dr. Israel Castellaños.)

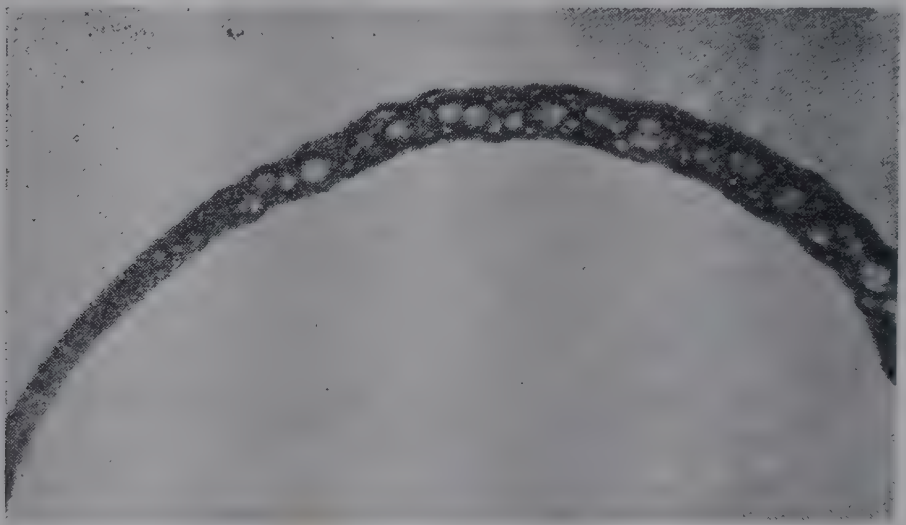

Fig. 656.—Here we see the bubbles which form inside of the hair when it has been exposed to a high temperature without burning. Hair submitted to excessive heat in a permanent wave by inexpert hairdressers has an appearance similar to this. (Courtesy Dr. Israel Castellaños.)

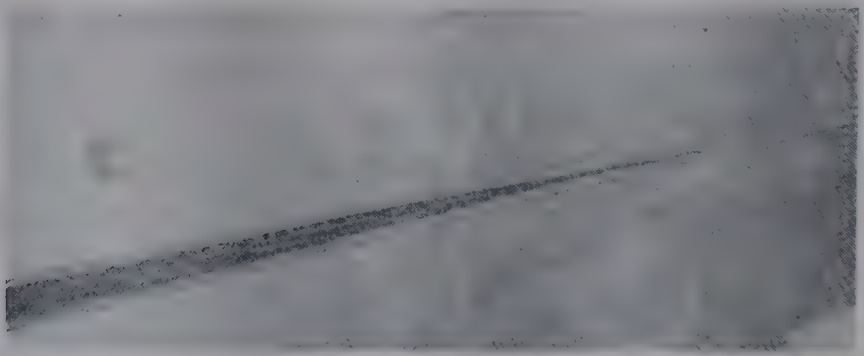

Fig. 657.—This is the point of a hair of animal origin. Note that the structural details of its central portion do not reach the distal end or free portion of the hair. (Courtesy Dr. Israel Castellaños.)

The intensive anthropologic investigations by Castellaños on the hair of Cubans, and his official position in a country where the inhabitants are accustomed to slaughter domestic animals, such as pigs, sheep, goats, etc., in the courtyards, especially on church holidays and national feasts, has shown the eminent importance of hair in police science.

Gross, the Father of Criminology, has insistently called the attention of scientific investigators and of the police to the importance of hair found at the scene of a crime. ''It is the duty of the magistrate,'' he wrote, ''to give his full attention to finding and delivering such hair immediately to experts, as a hair, in spite of its insignificance, may lead us to the discovery of the delinquent.'' (In Austria, the magistrate exercised direction over police investigation not paralleled in English-speaking countries. Dr. Gross was a magistrate.)

Criminal investigators pay considerable heed to the points made by Gross in his very valuable book. Castellaños does not accept his doctrine with respect to examination of hair, however. Gross suggested a simple procedure for establishing the position of the tip and the root of the hair. The method is efficient with respect to that object, but it requires cleaning the surface of the hair, eliminating the dust, dirt, etc., that has settled on it.

Care in Handling Hair.—Waldeyer, in his classic work, which appeared twenty years before Gross's ''Criminal Investigation'' was published, very appropriately observed: ''The dirt adhering to the hair is often more important than the hair itself.'' When a hair is found, therefore, it should never be rubbed between the fingers. Use of a magnifying lens will enable one, in most cases, to determine the direction of the tip or root of the hair.

It is of utmost importance in many cases not to touch any hair found if it can possibly be avoided. Gross recommended that the hair be placed on paper, fastened thereon by means of two glued paper strips, and the ends marked with the letters ''A'' for root, and ''B'' for point. At the same time a sketch is made and lettered accordingly, with letters corresponding to those of the specimen, so as to leave no doubt about the original position of the hair.

In countries like Cuba and America, where there is a mixed population, this may be difficult to do because all hair cannot be mounted in this fashion. Whereas the hair of most Caucasians can be mounted on paper in this manner, that of Mulattoes will seldom allow of it, and that of Negroes almost never. Hairs of these people are therefore preserved in clean white papers or in small envelopes.

Searching for Hair.—The smooth, lank hair of white persons and the straight, stiff hair of American Indians and Chinese are more likely to remain between the fingers of a victim than are those of other races. The more spiral the form of the hair, the less likely is it to stick between the fingers and be found in the hands of the victim, although his struggle with the aggressor may have been very fierce.

Because of the anatomic structure of its outer covering, hair sticks to clothes. The curlier it is, the tighter it sticks.

This brings to mind an interesting case reported by Chief Hugh D. Harper, of Colorado Springs, Colo., at the thirteenth convention of the International Association for Identification.

A white girl had been attacked by a Negro youth, and a suspect was arrested. The Negro people of Colorado Springs said that the accused man was innocent, the defendant's lawyer contending that the crime had been committed by a white man disguised as a Negro. The technical assistance of Dr. L. A. Bennet was requested. He found a long fair hair in the necktie of the youth, which he identified as having come from the raped girl. On the underclothing of the girl Dr. Bennet found pubic hair of a Negro, which he subsequently identified as having come from the arrested man. Thus the hypothesis of the defense was destroyed.

In America, where the population is composed of different racial types, knowledge of hair of various races is needed in police technic. The anatomic structure and the shape of the hair often indicate a definite ethnic type and thus facilitate police researches directed toward persons of some predetermined race. If, for example, a crime has been committed, and on the site of the crime one or more thick rigid hairs are found, it is assumed that a Chinese or an American Indian is involved. Likewise, if a kinky hair is found, some Negro must have been at the scene of the crime. Hair thus directs the investigation to persons of definite races.

One may admire beautiful atlases which form part of the most important works on hair. They do not, however, explain explicitly or precisely what ought to be done in police practice. There is no complete work on this subject.

Examination of the Hair.—It is advisable to keep the hair in bottles with ground glass stoppers in order not to lose its specific odor.

It is best to examine the hair as it is found. Pick it up with forceps and place on a slide. Cover with a cover glass. Do not touch the hair with the fingers, since this might remove any matter on the surface—dust, pollen, coal dust, etc.

Examine the hair under the microscope. Then, as Waldeyer recommends, without having cleaned the hair, add distilled water, and examine under constantly increasing magnification. One can often, in this way, discover blood, spermatozoa, pus, parasites, vegetable or metallic particles, etc., all of which can be identified. There are numerous instances where the hair is surrounded by substances which are found only on persons engaged in specialized occupations.

After having verified these preliminary observations, proceed to clean the hair with water, tissue paper, or a small brush.

Removal of Pigment.—If the hair contains much melanin, as is the case with Negroes and dark individuals, or if the medullary canals are full of air, it is difficult to observe the structural details of the hair or to determine the medullary index. To avoid this difficulty, authorities recommend removing the color from the hair by soaking it in nitric acid for several minutes if the examination is very urgent, or in oxygenated water for several hours if there is sufficient time.

In a great many cases the action of the nitric acid is protracted, and the hair under investigation is destroyed, arguments which might be brought out by either the prosecution or the defense. J. Delgado Roig, of Sevilla, Spain, has eliminated these difficulties by using oxygenated water and nitric acid; he uses a 15 per cent solution of sodium carbonate, in which he leaves slightly pigmented hair for half an hour; he leaves hair with much melanin for five hours in this solution. The hair is then mounted in Canada balsam, which is recommended by all authors. This method not only permits exact comparisons between two or more hairs, but also its identification at any later day, since the bleaching reagent does not impair or destroy the hair, even though it has been mounted for considerable time.

Cross-Section of Hair.—A portion of the hair should be cut in cross-section.

Cleanse the hair in 95 per cent alcohol for 5 to 10 minutes.

Immerse in 10 per cent sodium hydroxide solution for 5 to 10 minutes or longer depending upon how soon the hair becomes softened by the solution.

Immerse in a 20 per cent alcoholic solution of hydrochloric acid for 5 to 10 minutes.

Gonzalez suggests a convenient method of sectioning hair prepared as described. Partially incise a small segment of Formalin-fixed liver on one side, and stretch the hair out in the bed of the incision. The natural elasticity of the liver section will hold the hair in place. Then fix, harden, and embed the tissue in celloidin or paraffin. Cut it in the usual manner and mount the unstained section in balsam.

Gram Stain of Hair.—Stain with Gram stain, wash rapidly in 95 per cent alcohol, and mount in balsam without counterstain. The cuticula is outlined in violet wavy lines on the surface of the hair. If the hair is dark, it may be necessary to remove the pigment before staining, by bleaching it with a mixture of hydrogen peroxide and ammonia water.

Improved Technic for Sectioning Hairs. Method of Kirk[1]

It is often necessary and desirable to observe the hair in cross section. This is especially true when dealing with animal hairs. The transverse aspect is often highly diagnostic of a family with modifications in the genera. The usual methods of embedding lead to an angular section, which gives a distorted picture of the true contour of the fiber. Embedding in collodion is somewhat more successful, but the collodion-coated hair must ordinarily be mounted secondarily in paraffin to make it rigid. The problem of bending and distortion is therefore a serious obstacle to properly sectioned hair.

Kirk[1] described an improved method for sectioning hair. He advised a small microtome, developed by Hardy, with which a person after some experience can make a cross section in a very short period of time. The method combines embedding on a wood base with the use of a hand microtome which allows sections of 0.01 to 0.015 mm. in thickness or thicker.

Technic.—

Cut strips of balsa wood 1 cm. wide and 3 inches long from ¼ inch thick balsa planks.

On one of the centimeter-wide surfaces, apply a single layer of cellulose-acetate glue and spread out evenly to cover the wood strip completely in its central portion. Slow-drying "Comet" model airplane cement has been found to be readily available, convenient to use, and quite satisfactory as an embedding medium.

While the film of cement is still wet, lay the hairs to be sectioned on the cement parallel to the long axis of the balsa base.

As the film becomes tacky, apply a second layer of the embedding medium and spread so as to completely cover the hairs.

Label the block on the reverse side and place in a closed container, such as a small box, to dry slowly.

Observe two precautions. Do not apply more than the two necessary layers of cement because thicker films are much more difficult to cut; also do not accelerate the drying process since numerous and abnormally large bubbles are thereby formed which, if they surround the hair, tend to decrease the tenacity with which it is held. Upon drying, small bubbles are seen throughout the embedding medium. These do not ordinarily interfere with the quality of the final sections.

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[1] J. Crim. Law, Criminol. & Police Sci. 44: 124, 1953.

Sections may be cut within one-half hour if necessary, but they are thick and wedge shaped since the embedding cement is not completely dry, which tends to make them yield in front of the cutting edge. At least 12 hours, or overnight, is recommended as a satisfactory drying time.

Before embedding, clean and dry the hair, by washing thoroughly in acetone or alcohol-ether mixture and allowing it to dry on filter paper for a short while. This process takes only a few minutes.

If a number of hairs of a known source are to be embedded on the same block, they may be oriented while drying, with all proximal or distal extremities in the same direction. This procedure facilitates handling the specimen while affixing it to the block.

When thoroughly dry, place the wood strip carrying the embedded hair in the specimen clamp of a table model hand microtome (American Optical Model 900). Adjust the microtome until the portion of the hair that is desired for sectioning is even with the blade holder guides. A razor blade holder, designed for the instrument, is always used with a fresh sharp blade. Make the cut with a short slicing motion, through the embedding layers and hair, *but only as far as the underlying wood base.* Make the next cut in the same fashion, after advancing the block the amount desired. As many cuts may be made successively as required.

If another portion of the hair must be sectioned, adjust the block to the region of interest and repeat the cutting process. As the fiber is always in view through the cellulose-acetate layer, it is convenient to obtain sections close to the ends, in the center, or in any other area of the hair. The severed but unsectioned parts of the hair remain attached to the uncut block in their original relative position and may be recovered by dissolving away the lacquer with acetone. The block has suffered little damage and may be resurfaced with sandpaper and used repeatedly.

Using a pair of fine-tipped forceps, lift the hair sections, set in a slide of cellulose-acetate 0.5 to 1.0 mm. deep and 1 cm. wide, from the block and place in a small evaporating dish. Dissolve the lacquer with 1 or 2 c.c. of acetone. When the acetone evaporates, the resolidified cellulose acetate will be seen to have migrated up the sides of the dish, leaving the clean, free sections in the bottom.

Observe under a stereoscopic binocular microscope and transfer to a previously albuminized slide by touching each with the tip of a sharpened dissecting needle, to which they cling, then depositing them on the slide.

Inspect the slide briefly under the stereoscopic binocular microscope. Any sections standing on edge can be gently teased into their transverse plane with a needle.

A slightly faster procedure is to separate the lacquer slices and mount them on a slide. However, the lacquer slice surrounding the hair sections somewhat interferes with definition of the contour of the hair.

Apply a permanent mounting medium and cover slip. Clarite "X" dissolved in xylol is excellent. This medium infiltrates the hair and clears it very well for observation.

This technic has been useful in our hands. One difficulty we have had in getting the mounted hair in the proper position was due to the fact that we did not get all the lacquer off the hair before mounting it.* By using two or three washings with removal of the acetone each time, the lacquer is all removed.

Points in Investigation.—During the struggle which usually precedes the death of one or the other antagonists, it often happens that the hair or beard is grasped. Therefore, we must look for hair in the hands of the victim and on the clothes of the aggressor. If the victim died by strangulation, one must search not only the fingers but also the areas under the fingernails of the victim and the suspect. In cases of rape, the underclothing is examined. Most published cases refer to such findings.

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*Kirk, Paul L., Professor of Criminalistics, School of Criminology, University of California, Berkeley, Calif. Personal communication.

A review of the cases of death by shooting shows that the literature gives little information concerning investigation of hair in these cases. Investigators often do not look for hair or examine it in cases which appear to be suicides or homicides. They rely too much in certain instances on the necropsy findings. Forensic study of wounds and collection of data by the police should coincide, since police technic and forensic technic complement each other.

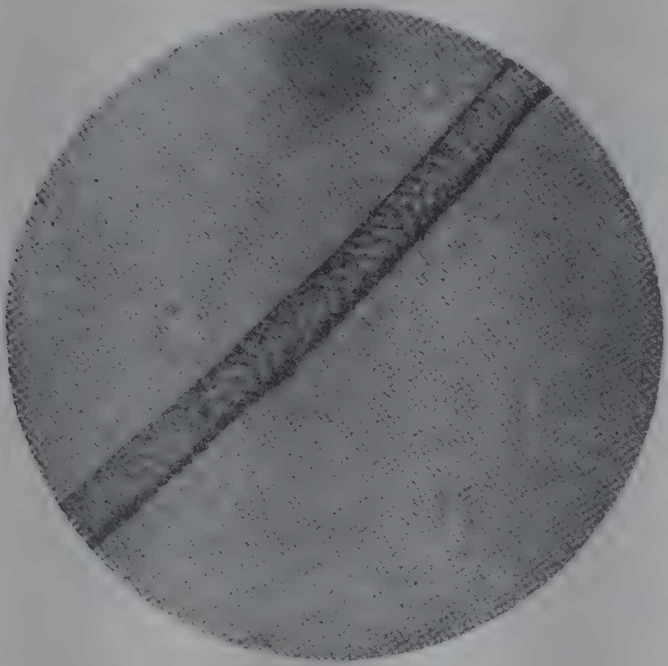


Fig. 658.—This is the hair of a sheep. The cuticular scales are discernible in no other type of hair. Note the absence of medullary channel and structural regularity of the cortex. (Courtesy Dr. Israel Castellaños.)

As an example of the part played by hair investigation, Castellaños cites the following case:

A woman, a foreigner, was found dead in a bathtub, in a state of advanced putrefaction. There were three large wounds on her head. It was alleged that she had died an accidental death, having slipped and fallen while bathing, and that her skull was fractured when it struck the faucets of the tub. Public interest in the case was great; some agreed with the accident hypothesis, while others insisted that this was a criminal death. Some days after the corpse had been found, Dr. Castellaños' bureau was called upon to make an investigation.

A thorough search was made and in the tub and on the walls of the bathroom, at approximately the height of the corpse, police recovered an abundance of hair. This was compared with that of the dead body and found to be identical.

The roots were examined; they presented evidence, without a doubt, of having been violently torn out of the scalp. Some of the shafts were bruised; others had been cut with a rather blunt instrument. The hair thus changed the situation, and led the police from the hypothesis of suicide to the certainty of homicide. To complete the demonstration, Castellaños proved that there was blood in the bathtub and that this blood had flowed from the victim; there was blood on the floor of the bathroom which had been washed; and, finally, on the basin, blood was found which the perpetrator of the crime had washed from his hands. When dressing before making his escape, he had left some gray hair between the teeth of a comb, which the Cuban Bureau is still holding. The medical examiner reported that a murder had been committed, and the police confirmed this thesis.

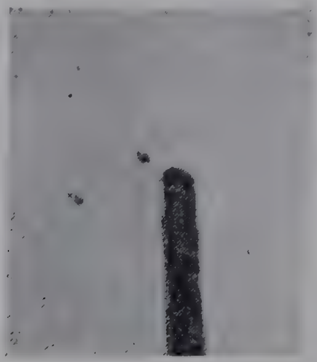


Fig. 659.—Round end of a hair shaft. This form appears on hairs which have been in continual contact with clothing. (Courtesy Dr. Israel Castellaños.)

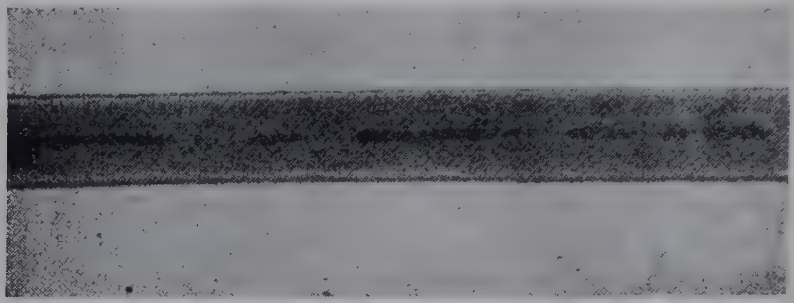


Fig. 660.—This is a shaft in which we can clearly observe the three constituents of hair—cuticula, cortex, and medulla. Note the lack of continuity of the medullary channel. (Courtesy Dr. Israel Castellaños.)

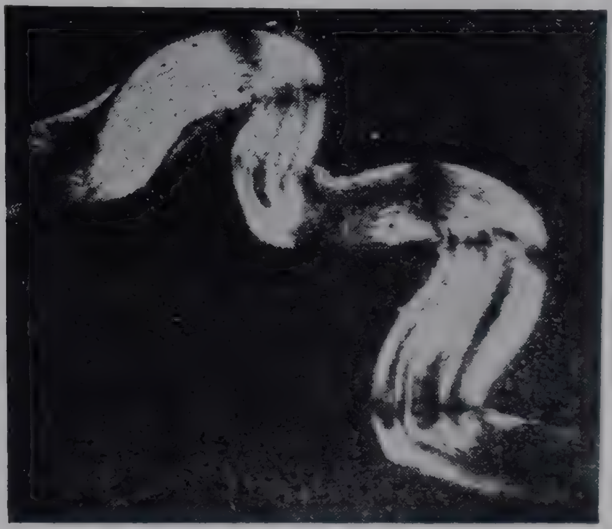


Fig. 661.—Photomicrograph showing the microscopic appearance of curled hair. (Courtesy Dr. Israel Castellaños.)

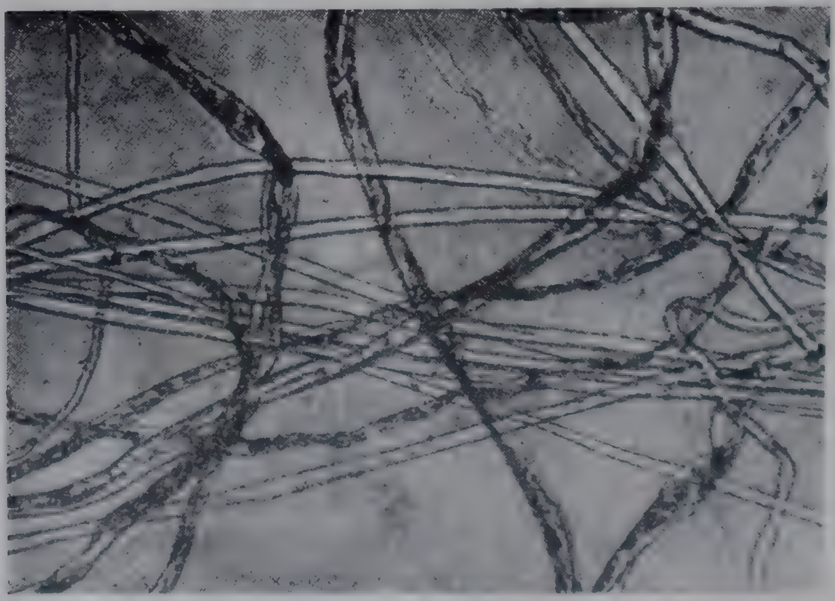


Fig. 662.—Cotton and silk fibers. Note the different microscopic appearance of each. (Courtesy Dr. Israel Castellaños.)

There are a great many homicides and murders by shooting which give the appearance of suicides. On the other hand, some cases of suicide present the appearance of murder. In these cases the study of the hair often helps to solve such medicolegal problems.

Effect of Firearm Wounds on Hair.—The first investigator to study the effect of firearm wounds on hair was Minakow. Daake, confirming the tests of Minakow, showed that the microscopic examination of hair is decisive in determinating the distance at which the shot was fired.

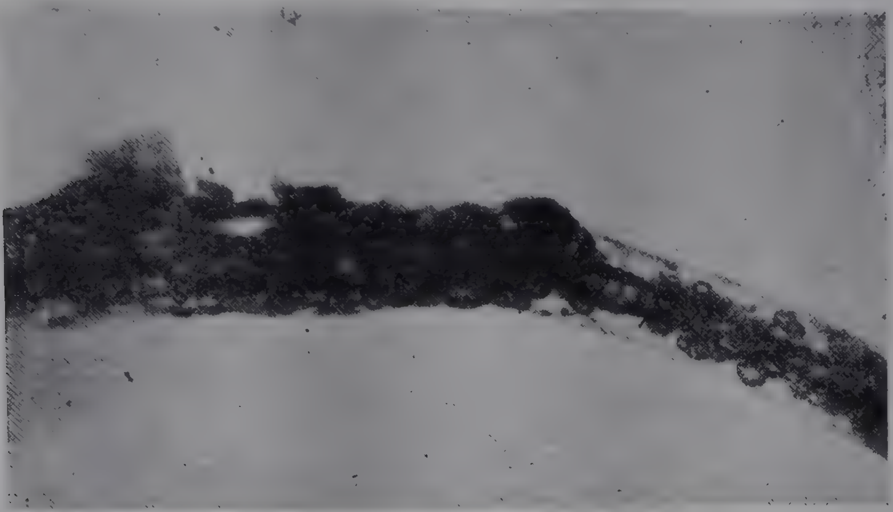


Fig. 663.—Appearance of the hair from a person who committed suicide by shooting himself. The hair is burned by a point-blank shot with black powder. Note the presence of bubbles produced by the heat in the mass of the medulla, and the residue left by the shot on the outside of the hair. (Courtesy Dr. Israel Castellaños.)

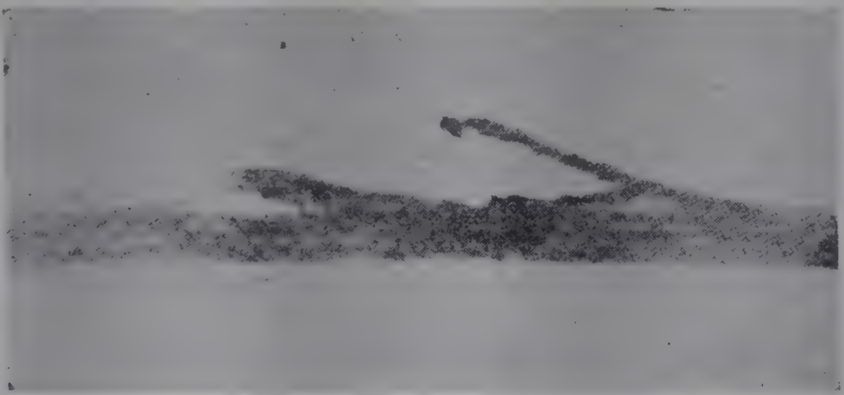


Fig. 664.—In this case the shot was fired from a short distance and smokeless powder was used. The cortex has been broken and raised by the unburned grains of powder, which always escape from the barrel of pistols or revolvers. Note that the outside of the hair also shows residue from the shot. (Courtesy Dr. Israel Castellaños.)

If the shot is fired from a very short distance, the hair of the victim is burnt. Microscopically the hair shows countless bubbles inside, but no change can be detected by the naked eye. Changes are greater after black powder has been fired than when smokeless powder is used. Schwalbe has performed magnificent researches in connection with this problem.

Temperature at Which Hair Burns.—It is very important to know the temperature at which hair burns. Piedelievre and Zebouni, using electric stoves, showed that at 100° C. hair shortens and loses weight; at 150° it presents gaseous bubbles in its medulla; and at 300° it is carbonized. These data are useful for determining to what temperature the body of the victim has been exposed. As an example, one case is cited.

In a village of the province of Santa Clara, Cuba, there lived a widow with her idiot daughter. The mother married a baker, and moved with her daughter into the baker's shop. The stepfather forced the idiot to carry logs for the oven, and in many ways was cruel to her. One day he beat her until she was almost dead. The poor idiot's cries were pitiful. After that incident she disappeared. The mother announced that she had left her home, but the neighbors were inclined to doubt her story and were suspicious that the baker had murdered her. An examination of the baker's quarters was ordered. Police searched the oven and found there countless carbonized hairs. Upon further examination and under questioning, the baker was proved to have murdered the girl and to have tried to destroy the evidence of his crime by burning the body in the oven.

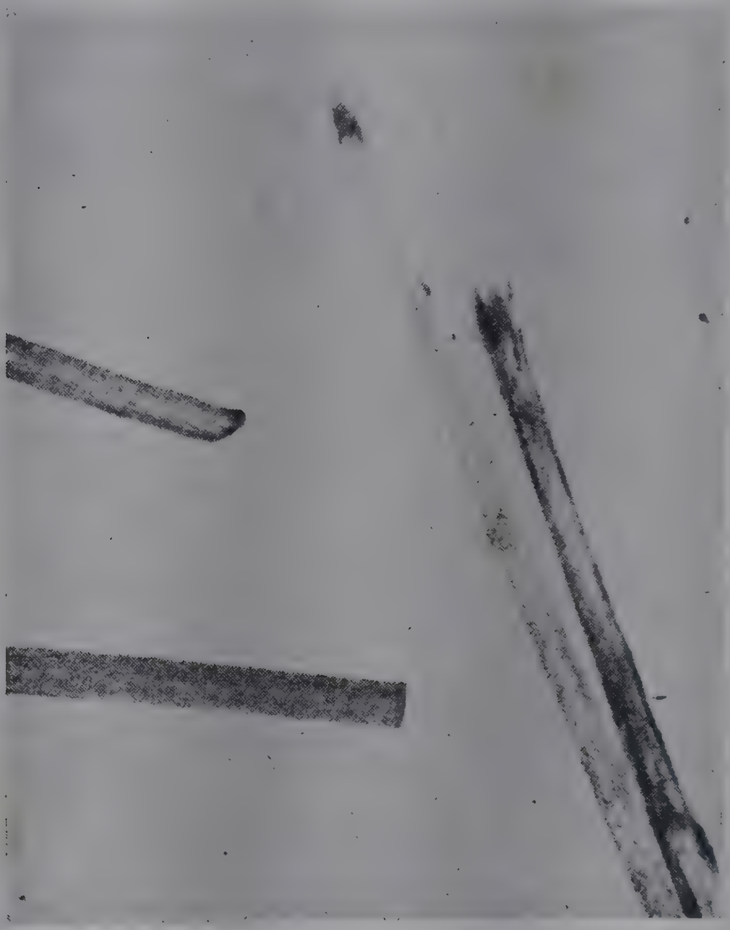


Fig. 665.—The point ending in a sharp angle and the one below, perfectly flat, with clean edges, were recently cut with the sharp scissors of a hairdresser. These hairs came from a woman. The lighter points, lying vertically, which are from less pigmented hair of another woman, are split, indicating the use of tweezers. The irregular or spread ends show that it has been a long time since the hair was cut. (Courtesy Dr. Israel Castellaños.)

Importance of Examining Hair.—All authors dwell at length on the different features of human and animal hair; the specific signs of hair cut with sharp instruments, of bruised hair, of hair torn out by violence, of hair which has fallen out spontaneously, in the course of its renewal; the details indicating the region or part of the body from which the hair comes; the data permitting deductions as to whether it belongs to a man or to a woman; the age of the person from whom it came; and the changes due to dyeing, curling, or combing it. These are but part of the program of identification. One must remember that hair, like bloodstains, reconstructs the crime in many cases, by confirming the correct and scientific findings of the autopsy, as in the case just cited. It may help to differentiate homicides from accidents; it helps to distinguish suicides and murders by proving the distance at which shots were fired; it offers definite proof in some cases of sodomy, as Kutter and Sonnenschein have shown; and it may determine the race of the victim or aggressor in even the most complex and difficult cases. For these reasons, a search for hair must be made in all criminal cases; it must be properly recovered and perfectly preserved, so that examination of such hair will give the best possible scientific results.

Hair should be searched for not only in some types of crimes of personal violence, as rape, strangulation, etc., where it obviously is likely to be found, but also in every crime, from robbery to murder, from infanticide to rape, from suicide to sodomy.

Microscopic Examination of Hair.—The importance of the microscopic examination of hair in criminal investigation has been described by many authors. Rolffs, in 1840, referred to the problem given the experts by a number of black hairs, obtained in 1832, in connection with a homicide. Orfila, in 1835, Ollivier, in 1838, Shuermeyer, in 1846, Falk, in 1856, Lender, in 1863, Kutter, in 1865, and many others thereafter have published cases and insisted on the necessity of studying the hair in order scientifically to decide a multitude of legal cases. In the introduction to the work of Glaister it is pointed out that, since 1851, British judges have considered hair to be valuable circumstantial evidence in criminal cases.

Hair is a distinctive specialized anatomic structure. It consists of a shaft, a point, and a root. That part of the hair which is not embedded in the skin is called the **shaft.** The shaft terminates in a free end called a **point.** The part which is inserted in the skin is called the **root.** The root swells into a more or less ovoid form called the **bulb.** The bulb contains an indentation, very much like the bottom of a bottle, in which is contained the **papilla,** a projection of the skin, which is the organ which produces the hair. Each of these parts—the shaft, the point, and the bulb—offers important details. We may compare a hair with a fine pin, in which the head is the bulb, the body is the shaft, and the sharp extremity is the point.

The shaft is composed of the **medulla,** the innermost core of the hair; the **cortex,** the intermediate layer which surrounds the medulla, and which is the thickest and most distinctive part of the shaft; and the **cuticula,** around the cortex. The medulla looks like an irregular cylinder and contains air spaces and some scattered cells of various sizes and shapes. The medulla often disappears toward the distal end of the shaft. The very fine type of human hair might not contain a medulla. The cortex is composed of elongated, spindle-shaped, longitudinal, horny plates attached closely to each other. They contain pigment granules. The cuticula is composed of non-nucleated overlapping polygonal cells, the free edges of which are directed toward the distal end of the hair.

Age of the Individual From Whom Derived.—Oesterlen gives the following average diameters of hair of different age groups:

12 days	24 microns
6 months	37 microns
18 months	38 microns
15 years	53 microns
Adults	70 microns

Lanugo hairs on the fetus are very fine, have no medulla, and are almost colorless. The average diameter of the hair increases with age as can be seen in Oesterlen's table. The hairs of infants and children are usually finer than those of adults, they usually have no medullary cavity until after puberty, and contain less pigment than do adult hairs. Gray and white hairs appear in middle life. This is due to loss of pigment from the shaft. Gray hair without pigment is usually translucent or may even be transparent. The diameter of the hair in the aged is less as a rule than that of younger adults.

It was formerly thought that the morphology of the medulla of human hair depended on the age of the individual, but E. M. Wynkoop showed that this is in proportion to the diameter of the shaft of the hair and is not due to age.

The hair of young people has a lower pigment content and a smaller diameter than that of adults. In elderly people the hair tends to become scarcer and finer and the bulb degenerates. The shaft also shows signs of degeneration.

Race.—Scalp hair is usually examined to study race. The color of the hair of the Caucasian race varies from light to dark. The shaft may be fairly straight or wavy and curly with varying caliber throughout the length. The shaft is ovoid. Medulla is central in straight hairs or slightly excentric in curly hair. In dark individuals of the Caucasian race the cortex contains much pigment and is dark brown. In fair individuals the hairs are lighter brown, or they may be reddish or blond.

American Indians, Chinese and other Asiatics usually have long straight coarse black hair with deeply pigmented large shaft. Cross sections show cylindrical or triangular shafts with the medulla centrally situated.

Hair of Negroes is usually kinky and black, the caliber varying greatly along the entire length of the hair. A large amount of pigment is to be found in the cortex. The hair in cross-section is ovoid and narrow or may be almost flat. The medullary canal is excentrically placed.

Sex.—It was formerly possible in many cases to identify hair from female subjects by the length of the hair, but since women cut their hair frequently today and also shave the armpits and legs, the length and shape of the point are of much less value than formerly for differentiating hair. The best rule, as indicated by Glaister, is that a woman's hair generally shows a more delicate structure and a much finer granulation of pigment than that of a man. A study of the cross-sections shows that the sexual difference in the weight of hair is due to the difference which exists in the area of cross-section and the difference in the amount of pigment. M. Bernstein and S. Robertson, of Chicago, showed that male hair is heavier than female hair.

Region From Which the Hair Is Derived.—To determine the region from which the hair is derived, consideration is given to the length, the diameter, the shape of the point, the material which covers the surface, and the shape of the cross-section of the hair. Hairs from the beard are usually larger and curlier than those from the scalp. They usually show the effects of shaving, although if undisturbed will taper to a point. Hairs from eyebrows, eyelids, nose, and ears are as thick as the hair from the scalp but are much shorter, and taper rapidly to a point. Medullary cavities are consistently present. Hairs from the axilla and pubic regions are fairly long and uneven with excentrically placed medullary canals. The hair has a tendency to curl. Hairs on the trunk and extremities vary in thickness and have less pigment than other hairs. There is a fine medullary cavity, although this may be absent.

The mere presence of certain substances indicates the region from which the hair came. A hair covered with fatty matter unquestionably came from the armpit; a curly hair, with crystals and vaginal secretion, can come only from the genital region of a female, etc.

Glaister summarized the bases of diagnosis as follows: (1) the total length of the hairs, (2) their texture and contour, (3) the character of both ends, (4) the general thickness, (5) whether sweat, vaginal secretion, seminal fluid, fecal material, food, nasal secretion, or wax adheres to them, (6) the exact character of the cross sections.

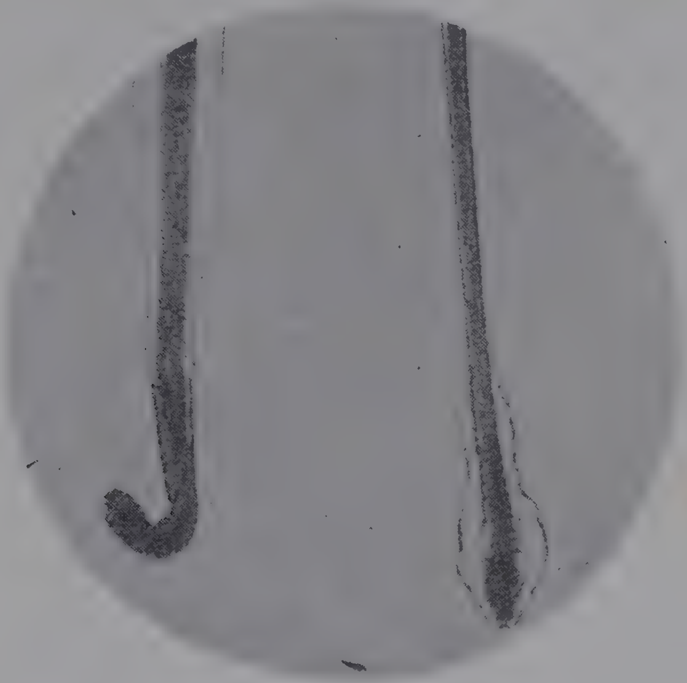


Fig. 666.—Root portion of hairs which have been torn out. Note the typical appearance of the bulb. The one at the right carried part of the follicle sheath. (Courtesy Dr. Israel Castellaños.)

Miscellaneous Facts.—Hair which has been recently cut shows the cut end more or less clean, according to the sharpness of the instrument used. At times hair is found in caps or hats with both ends cut, one cut being recent and the other much older. In all cases where the cut is recent, the edges are clean, neat, and form more or less acute angles. After three days the point of the cut hair begins to become rounded, convex, because of the continuous rubbing of substances against it.

Pincus estimated that an individual of 20 years loses 70 hairs of his head daily; this figure decreases until the age of 50 years, when only 22 hairs are lost each day. Hair which falls out of itself shows a full bulb, replete, as it has reached full growth. On the other hand, those which have the bulb hollow or excavated, because they have not attained full growth, show that they were pulled out. When the pull has been very violent, the root is found to have particles or cells of the surrounding skin clinging to it. In some cases hair which has been pulled out is found to have numerous cells of the skin adherent to the root, and the shaft is more or less noticeably bruised. This always occurs when the victim was attacked with a heavy, blunt, and crushing instrument.

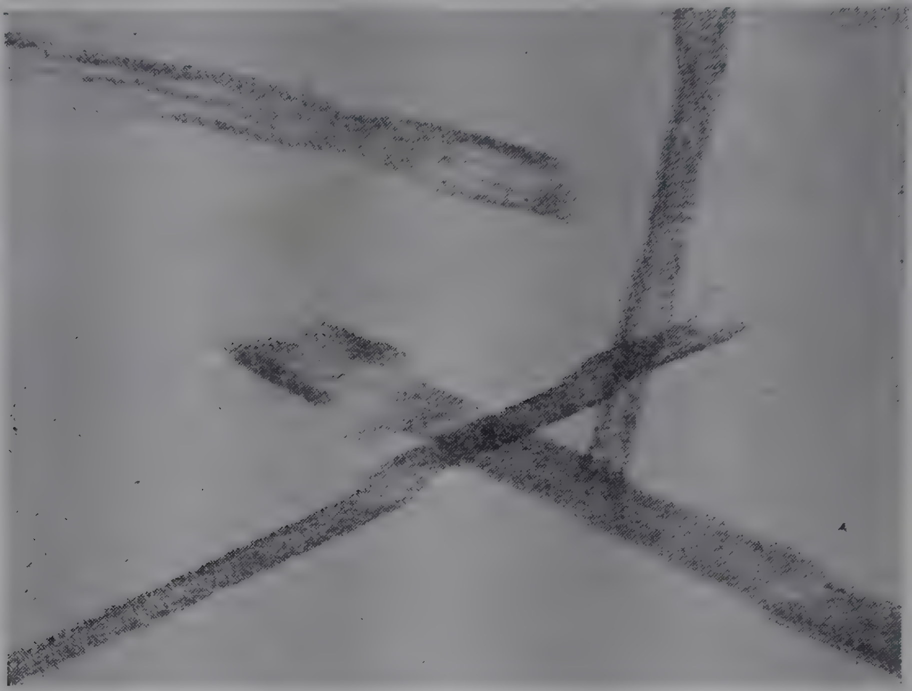


Fig. 667.—Microscopic appearance of several hairs which have been struck with an instrument or weapon without a cutting edge. It can be seen how part of the hair shaft is bruised. (Courtesy Dr. Israel Castellaños.)

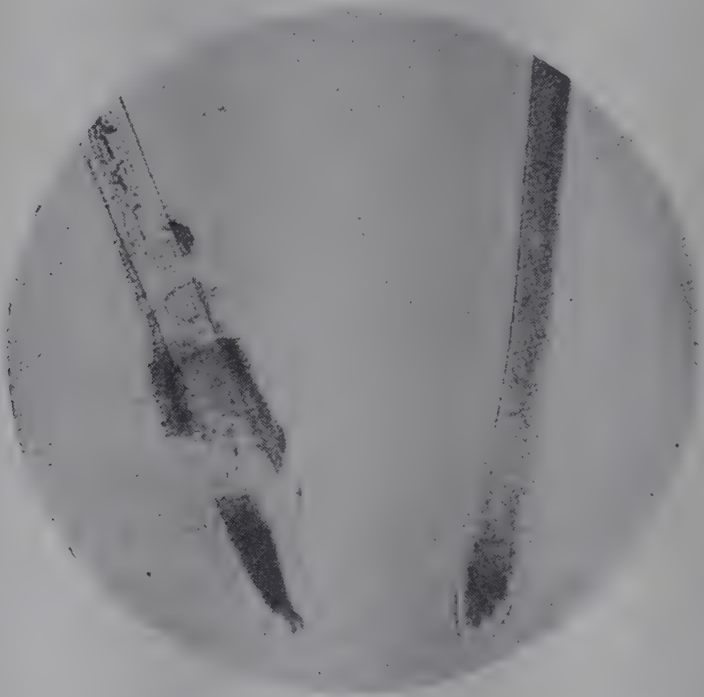


Fig. 668.—Root portions of two hairs. The one at left was torn out and the one on the right fell spontaneously. Note the typical appearance of the terminal portion in this fallen hair. (Courtesy Dr. Israel Castellaños.)

Bleaching or Dyeing the Hair.—Bleaching of the hair is more difficult to determine than the fact that the hair has been dyed. Dyes are easily discovered, because they leave on the periphery of the hair a homogeneous or irregular layer which can be seen perfectly under the microscope. Tests for determining the dye used are based more on chemical analysis than on microscopic examination.

Dyes of a metallic salt base, bismuth, silver, or lead, or those having a base of organic substances, such as vegetable dyes, can be determined by chemical analysis.

Many criminals do not hesitate to make use of surgical means to disfigure themselves and thus evade the action of the police; they may also use wigs for that purpose. It is necessary, therefore, to be able to distinguish between hair which has recently fallen out and false hair.

Examination.—If, in the hands of the victim, one finds hairs which the victim pulled from the head or beard of his aggressor during a struggle, note must be made of the position of the hairs in the victim's hand. For greater exactitude, as Gross suggested, a drawing is made of the hand to fix that position exactly.

It is often possible to tell the root at a glance, by the enlargement of the bulb, which is always visible with an ordinary magnifying glass. It is then unnecessary to take steps to decide where the point lies and where the root is.

The material adhering to the surface of the hair should not be dislodged, since, as Waldeyer stated, "often the dirt adhering to the hair is more important than the hair itself." For this reason, any hair which may be found must be neither rubbed nor cleaned.

The hair from the victim should be placed in bottles labeled "victim," and any hair from a suspected aggressor should be placed in bottles labeled "suspect."

When no hair is found in the hands of a dead body, and none is found at the scene of the crime or in the place where the victim was found, search should be made of articles of personal use, such as towels, combs, etc. Hair commonly adheres to clothing, not only to caps and hats, but also to stockings. It can frequently be found in the nap of towels, and it may be the hair of the victim or of the aggressor. It is rare not to find hair in the teeth of combs. Such hair may be from the deceased when he last used the comb, or it may have come from the aggressor if he combed his hair before leaving the scene.

Hair may also be found on sheets and pillow cases. It consists of hair which has fallen out while the victim was resting or sleeping in the bed, or hair which has been pulled out violently in the struggle which preceded death.

Hair comes out not only because of the periodic renewal to which it is subject, but also because of a tug given by an individual in defending himself or by the aggressor in an attack. The hair falls unnoticed and remains at the scene of the crime as a silent witness to the tragedy and as circumstantial evidence of the crime. If the investigator searches for it with a magnifying glass, he will find that it contains a varied number of substances, according to the nature of the crime: blood from a mortal wound, black or smokeless powder from a fatal shot, semen, etc.

Finding a hair, according to Glaister, makes it necessary to answer these questions:

1. Is the hair that of a human being?
2. If it is human hair, is it the hair of a male or a female subject?
3. What is the approximate age of the individual from whom the hair has been derived?

4. From what part of the body has the hair been derived?
5. Has the hair been dyed or is it of natural color?
6. Has the hair fallen out, been pulled out, or cut?
7. If it has been cut, was a sharp or a blunt instrument used?

Lambert and Balthazard suggest the following program:

1. Is the hair that of a human being or of an animal?
2. From what part of the body does the hair come?
3. Is the hair from a male or female individual?
4. What is the age of the individual from whom the hair came?

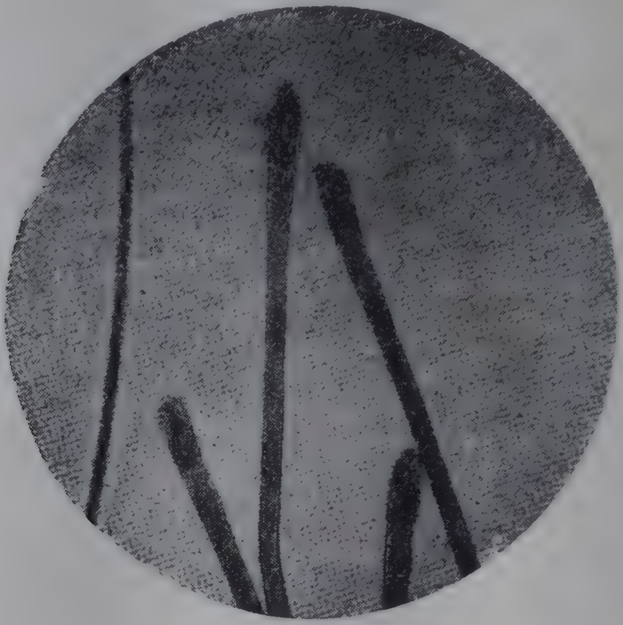


Fig. 669.—These hairs show that in fallen hairs the root sheath is missing. In cases of falling hair, part of this sheath always remains embedded. (Courtesy Dr. Israel Castellaños.)

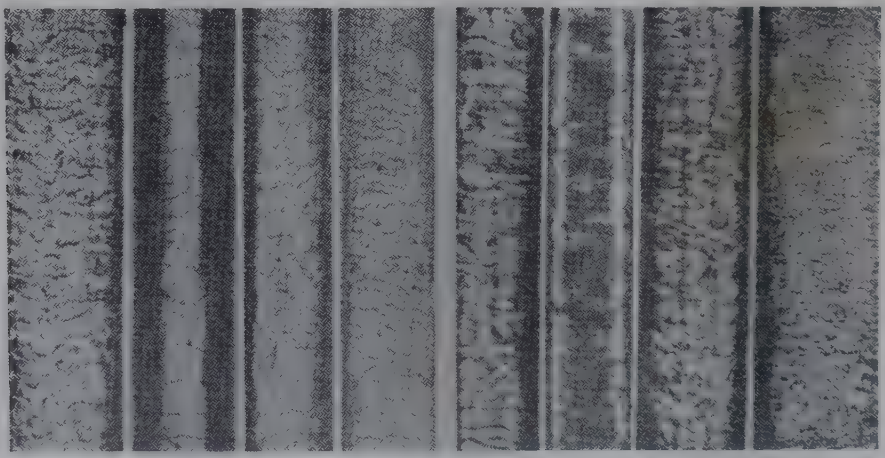


Fig. 670.—Hair characteristics. Left to right, hair of Negro, Mulatto, white man, white woman, dog, cat, horse, and pig. (Courtesy Dr. Israel Castellaños.)

5. Was the hair cut, and at what time?
6. Was the hair pulled out, or did it fall out accidentally?
7. Is the hair dyed, and if so, by what process?
8. Did the hair under examination come from a living person?
9. The identification of the hair.

Castellaños' questionnaire is longer, because he makes additional examinations to determine:

a. The trade of the individual from whom it came.
b. The distance at which the fatal shot was fired in cases of gunshot deaths.
c. The kind of powder used in the fatal shot in such cases.
d. The poisoning of the subject from which it came.

Human and Animal Hair.—Most authors begin the investigation by determining whether the hair found is from a human subject or from a lower animal. At present there is no one known identifying specific characteristic of human hair, just as there is no illness which can be diagnosed by a single symptom; but the hair of no one animal combines the various anatomic characteristics found in the hair of man. Table 215 gives the characteristics of human and animal hairs. See Figs. 655, 657, 658, 670.

TABLE 215.—CHARACTERISTICS OF HAIRS COMPARED

A. Medullary (Center) Channel

I. MAN

a. Aerial net with fine granules
b. Invisible
c. Medullary cells not previously dissociated
d. Value of medullary index (relation of diameter of the medulla to shaft diameter) inferior to 0′ 30
e. Downy hair without medulla

II. ANIMAL

a. Aerial content of vesicles more or less voluminous
b. Medullary cells very apparent
c. Value of medullary index over 0′ 50
d. Medulla in steps in the downy hair

B. Cortical (Outer) Substance

I. MAN

a. Constitutes a thick muff
b. Pigment in very small homogeneous granulations

II. ANIMAL

a. Constitutes a very thin tube
b. Pigment in irregular granulations, always larger than those of man

C. Cuticula

I. MAN

Thin scales, lightly serrated (saw-toothed), strongly imbricated (tile-like)

II. ANIMAL

Thick scales, less imbricated than in man, markedly serrated.

Microscopic Comparison of Hair.—It is after having settled all the points discussed above that the investigator undertakes what may be properly called the identification of the hair, that is to say, the individualization thereof, or, in other words, the determination of whether the hair found comes from a definite suspect or from a given individual.

Measurements are taken of the length of the hairs under comparison and their average diameter, and the maximum measurements are noted. If the different data coincide and the pigmentation of the hair is identical, as determined by the Fischer chromatic scale, one has reason to believe that the origin is the same. To make the examination definitive, minutely study the individual signs or characteristics of the hair. Observe the peculiarities of the *medullary canal* and the *point*. The coincidence of wear or degree of rubbing of the free point of the hair, with the coincidence of the length and the coincidence of the diameter, consolidate the identifying diagnosis. The coincidence of arrangement, abundance, and coloring of the pigmentary granulations make identification or individualization of the hair certain.

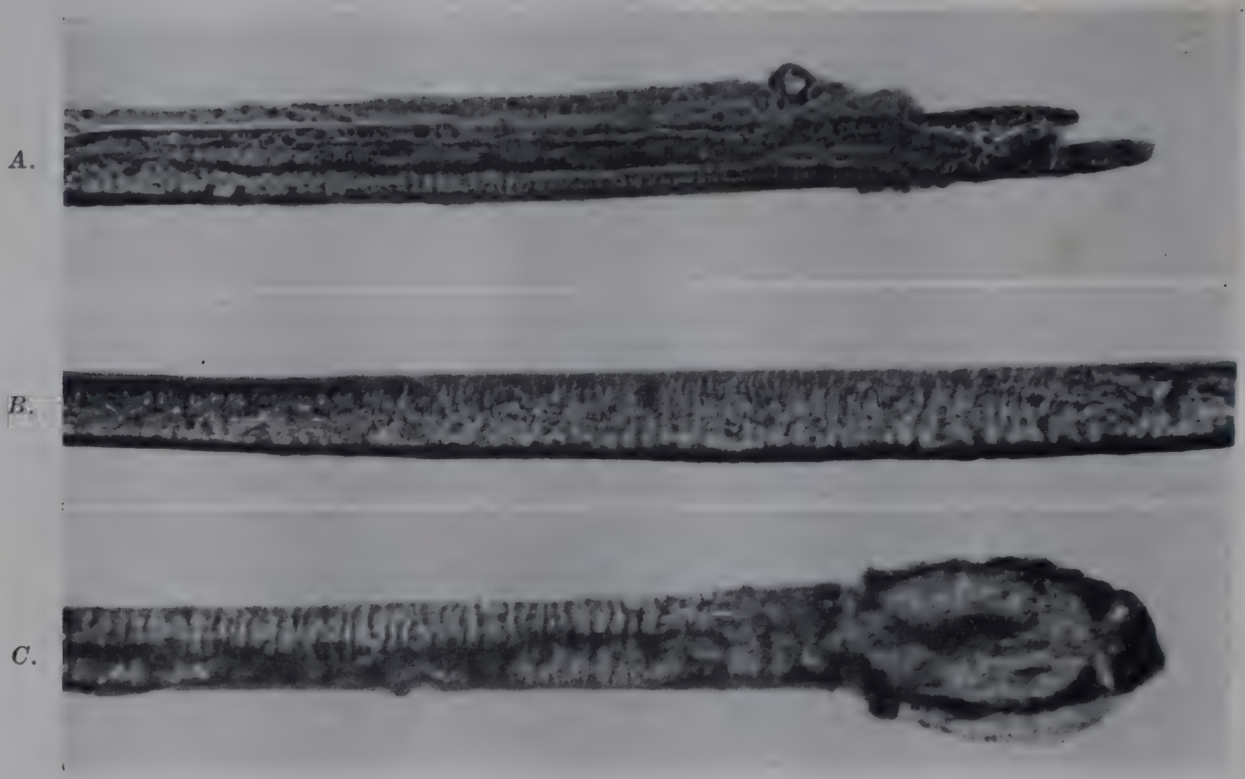


Fig. 671.—Exhibit A, Case 1. *A*, Photomicrograph of the severed end of the hair found in the slit of a screen door. *B*, Photomicrograph of the middle portion of the same hair. *C*, Photomicrograph of the root of the same hair. (Courtesy Dr. Israel Castellaños.)

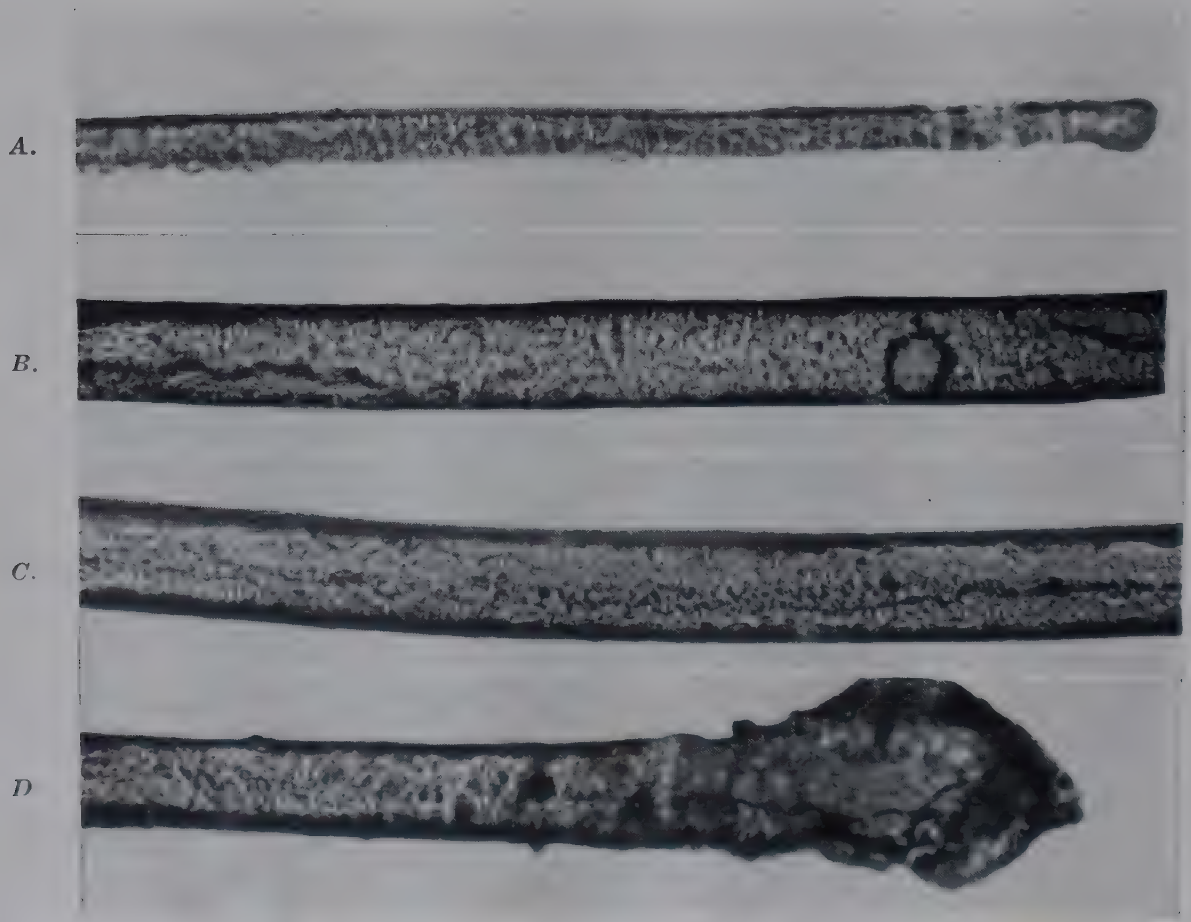


Fig. 672.—Photomicrographs of one of the hairs taken from the left arm of suspect B, Case 1. *A*, End portion of the hair. *B*, Portion near the middle of the same hair. *C*, Portion beyond the middle of the same hair. *D*, Root of the same hair. (Courtesy Dr. Israel Castellaños.)

In addition to these details, purely anatomic, there are many others which contribute to make identification of the hair more eloquent; such as racial characteristics, the existence of identical vegetable substances, metal particles, pathologic changes, parasites, etc.

The presence of foreign particles, such as coal, pollen, etc., may prove to be indications of the trade in which the individual was engaged.

Finally, it has been shown that with adequate chemical methods, presence of arsenic in the hair can be shown when the individual has been poisoned with arsenicals.

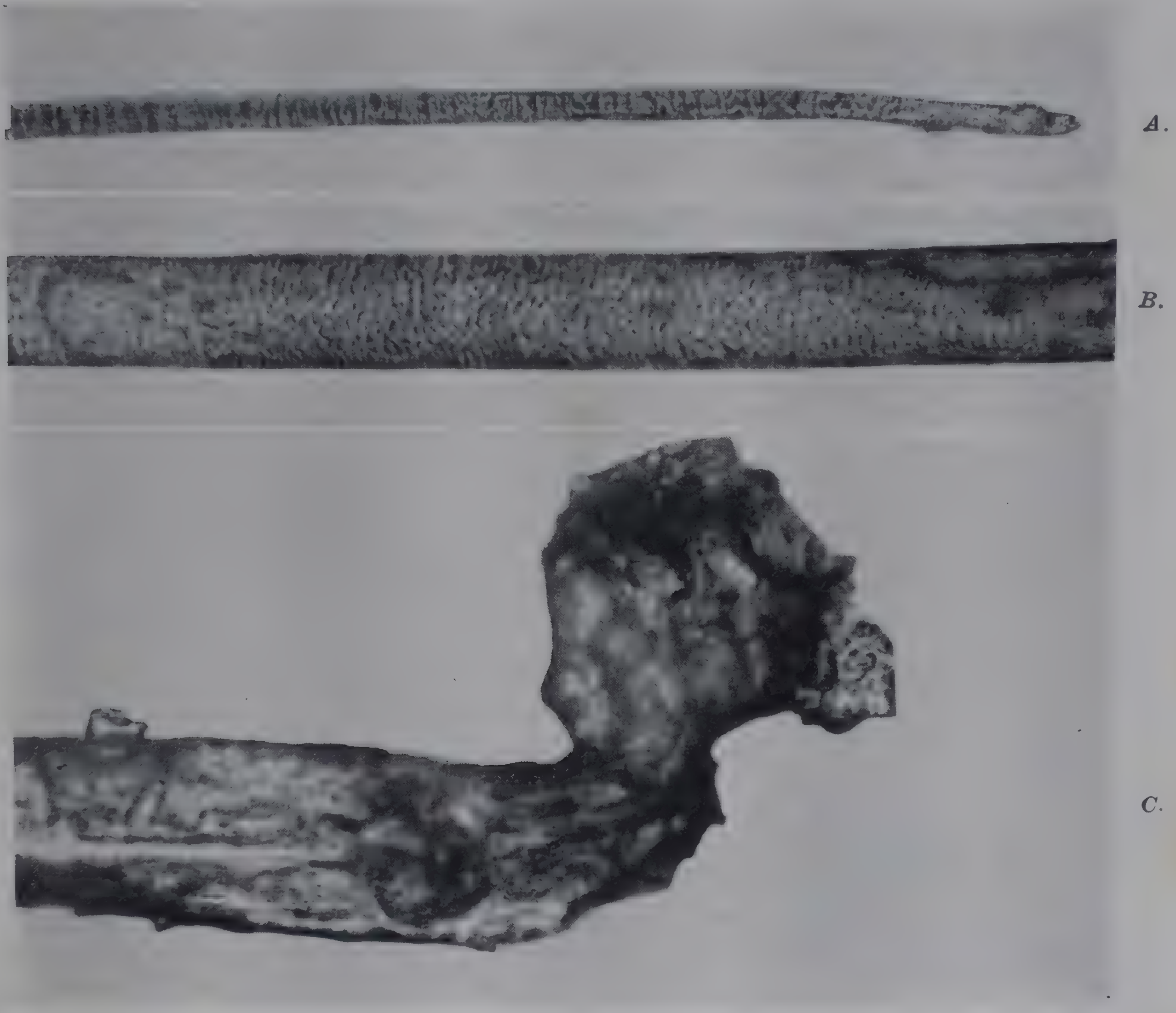


Fig. 673.—Photomicrographs of one of the hairs taken from the left arm of suspect C, Case 1. *A*, End of the hair. *B*, Middle portion of the same hair. *C*, Root of the same hair. (Courtesy Dr. Israel Castellaños.)

It may be concluded that microscopic, chemical, and bacteriologic study of hair offers a fertile field of criminal investigation. From Castellaños' experience of over fifteen years he has found, on numerous occasions, that when the usual investigation had failed and the criminal seemed about to be freed, he was able, from a few hairs which he had obtained in some cases weeks after the tragedy, to identify the persons involved in the crime and to deliver the guilty to justice.

In a personal communication, May, 1947, Dr. Castellaños furnished the author with records taken from his own files of cases in which the identification of hair contributed an important part to a medicolegal procedure.

Case 1.—In Louisiana, the screen door of a camp hut was found broken. Investigators found a hair caught in the broken screen wires and, using the necessary precautions, took it for examination. Specimens of hair were also taken from suspected persons, those near the camp hut during the night. These were submitted for comparison.

The hair taken from the slit in the screen door was labeled ''Exhibit A'' (see Fig. 671 *A, B, C*). Those from the two suspects were labeled, respectively, ''Exhibit B'' and ''Exhibit C.'' Examination of Exhibit ''A'' established:

1. That said hair is human hair.
2. That it came, not from the head, but from an extremity.
3. The color of the hair is light blond.
4. The free end of this hair appeared to have been cut transversely by an edge which was rather thick and not extremely sharp. If the hair had been cut by a very sharp edge, the free end would show a straight, clean cut instead of the jagged cut as seen in Fig. 671, *A*.
5. Examination of the full length of this hair revealed that the stem was not injured or compressed, since all of its elements were intact. The regularity of the hair and the integrity of the cuticula can be seen in Fig. 671, *B*. The diameter varied from 22 to 56 microns.
6. That the root of this hair presented all of the characteristics found in hairs which have been pulled out, as is seen in Fig. 671, *C*.

Examination of Exhibits ''B'' and ''C'' showed:

1. That these are also human hairs taken from the extremities.
2. That Exhibit ''B'' comprised nine hairs the length of which ranged from 8 to 28 millimeters.
3. That Exhibit ''C'' comprised five hairs the length of which ranged from 11 to 27 millimeters.
4. That the diameter of the hairs in Exhibit ''B'' varied from 14 to 66 microns.
5. That the diameter of the hairs of Exhibit ''C'' varied from 12 to 134 microns.
6. That the hairs taken from Exhibit ''B'' were light like those of Exhibit ''A,'' while the hairs taken from Exhibit ''C'' were darker or pigmented.
7. That the similarity of color of the hairs of Exhibits ''A'' and ''B'' was clearly visible, and microscopic comparison proved that the tone or chromatism of both exhibits was identical.
8. That while the disparity between Exhibits ''A'' and ''C'' was clearly visible to the naked eye, microscopic comparison proved the chromatic discordance between these exhibits.
9. That through macroscopic and microscopic examination, and the micrometric measurements taken of the nine hairs sent as Exhibit ''B,'' their anatomic structure, characteristics of cuticula and outer covering, the nature of the medullary structure, the tone and arrangement of pigmentation, proved that they were identical to those of Exhibit ''A.''
10. That through all of the aforementioned anatomic details it was proved that the five hairs sent as Exhibit ''C'' were completely different from these of Exhibit ''A.''
11. That all the scientific observations made proved that all the macroscopic, microscopic, anatomic, metric, and chromatic characteristics found in Exhibit ''B'' are also found in Exhibit ''A,'' while the contrary is true of Exhibit ''C.''
12. That all these data coincided to establish the fact that the hairs taken from the left arm of Suspect No. 1 were exactly the same as those taken from the hair found in the slit in a screen door in the rear entrance to a camp hut. Fig. 674 shows that the hair of Exhibit ''A'' coincides exactly with that of Exhibit ''B'' as registered by examination with the Spencer comparison microscope.

CASE 2.—A man was struck and killed by a motor vehicle. A passenger bus had been seen in the vicinity at about the time the man was killed. The driver of this bus was questioned by the police. He stated, under questioning, that he had felt a slight jar at about the place where the body had been found. It was quite dark. He had stopped his bus, looked around and, finding nothing wrong, had driven on.

An examination of the bottom of the bus revealed the presence of one strand of hair and a small fragment of what appeared to be human tissue.

Later a quantity of hair was taken from the dead man's head for comparison.

Castellaños received two specimens marked, respectively, ''Victim'' and ''Questioned.'' The envelope marked ''Questioned'' contained the tiny fragment seen in Fig. 675.

This tiny fragment, when placed upon slides, showed two objects, the details of which may be noted in Fig. 676, *A* and *B*.

The dirt was removed from the small organic fragment by washing it in saline solution which does not alter the anatomic structure or the color. The cleansed fragment was then prepared for microscopic examination, which revealed that the fragment was connective tissue, as seen in Fig. 677. The preliminary microscopic examination revealed that the material clinging to the small fragment was hair and not fiber.

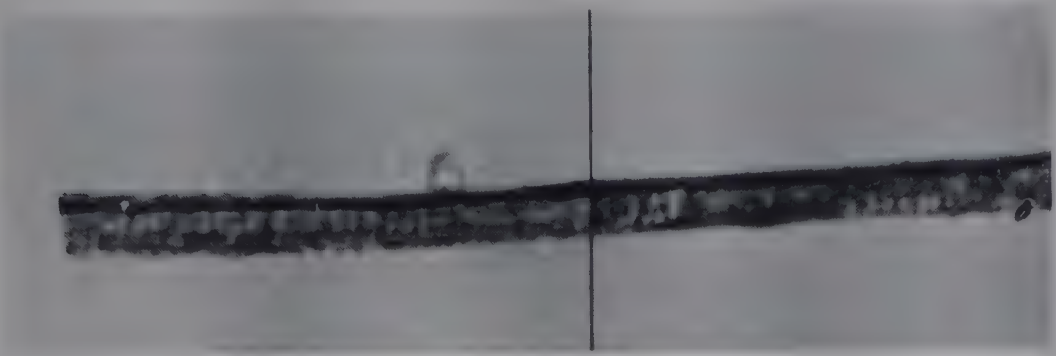


Fig. 674.—Photomicrographs of Exhibit "A" and "B," Case 1, as seen under the Spencer comparison microscope. (Courtesy Dr. Israel Castellaños.)

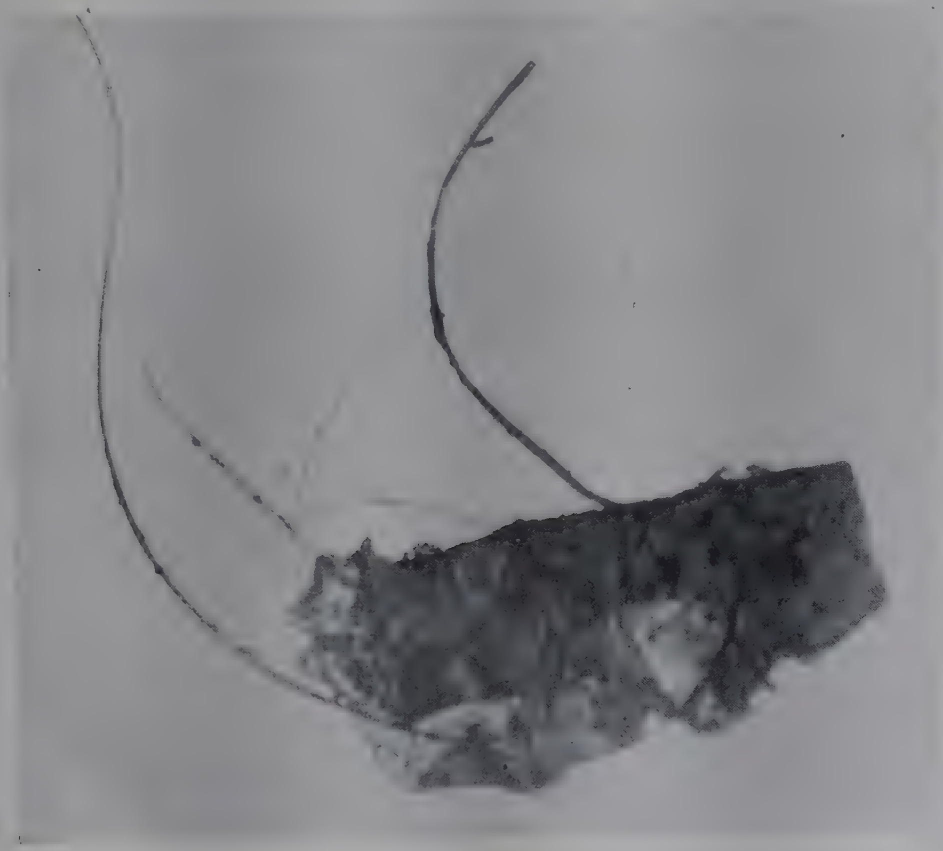
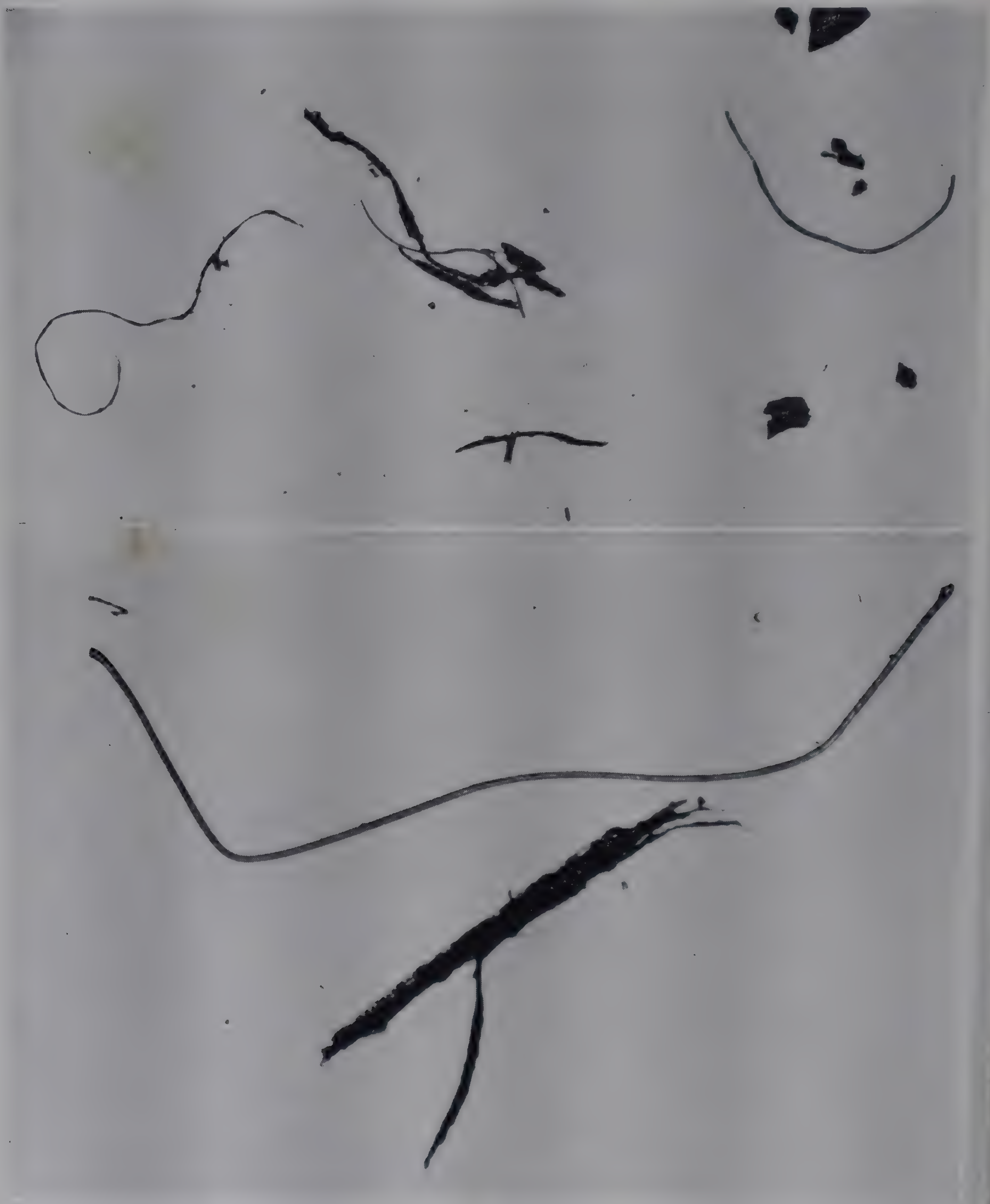


Fig. 675.—Tiny fragment contained in envelope marked "Questioned," Case 2. (Courtesy Dr. Israel Castellaños.)

As can be seen in Fig. 676, *A* and *B*, the hairs which were clinging to the fragment were dirty. Before they could be submitted to complete anatomical examination, they were thoroughly cleansed in saline solutions. Saline solutions make not the slightest alteration in the structure or coloring of hair. These hairs, removed from the connective tissue, were 1 to 2 cm. long and were arched.

The hairs terminated sharply or in a point. The stem or body of the hairs was bruised, crushed, or broken. Fig. 678 shows a crushed portion on the right-hand side. There are portions or fragments, however, in which the structure is normal, as seen in Figs. 679 and 680, *A* and *B*.

The hairs removed from the small piece of connective tissue were examined under natural and transmitted light and classified according to the Bertillon chromatic classification as "light brown."



A.

B.

Fig. 676.—*A* and *B*. Contents of envelope marked "Questioned," same as Fig. 675. (Courtesy Dr. Israel Castellaños.)

The cuticula of these hairs, especially in the stem or body, was definitely imbricated. The end, which is the portion opposite to the root, was disintegrated; parts were lacking so that the cortex was exposed. Such disintegration shows that these hairs had been altered mechanically by repeated friction and crushing. The stem of one of the hairs showed a green-stick fracture, in which the body of the hair is split open. The medulla was disunited in some regions and in others it was not visible but showed a granular airlike network.

The examination of the diameters of the shortest hair, approximately one centimeter long, showed: maximum diameter, 98 microns; minimum diameter, 17 microns; average diameter, 56.1 microns; medullary index, 0.25.

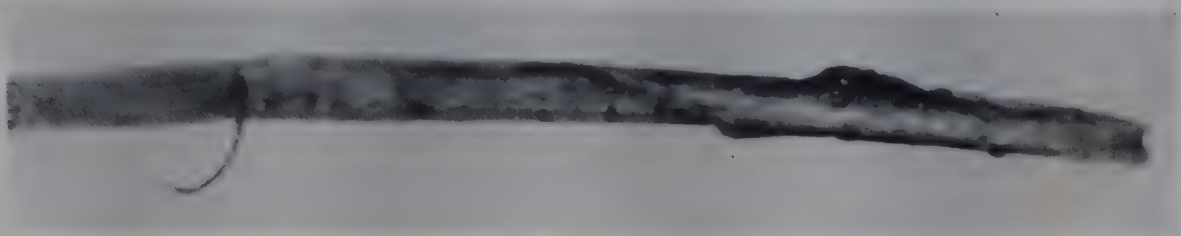


Fig. 677.—Enlargement of fragment (Fig. 676), which reveals it as connective tissue. (Courtesy Dr. Israel Castellaños.)

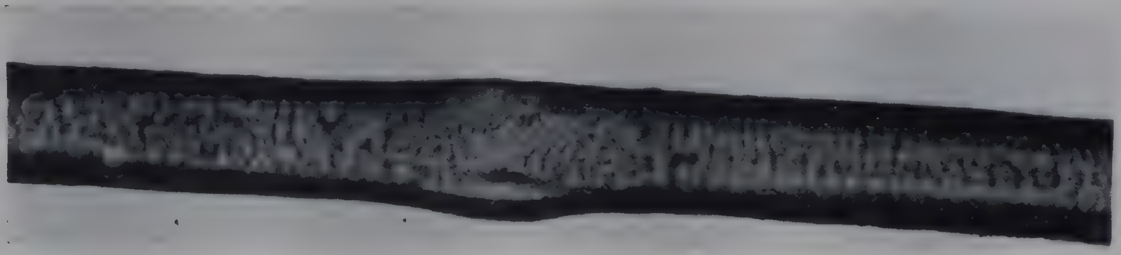


Fig. 678.—Body of hair showing crushed portion on the right-hand side. Case 2. (Courtesy Dr. Israel Castellaños.)

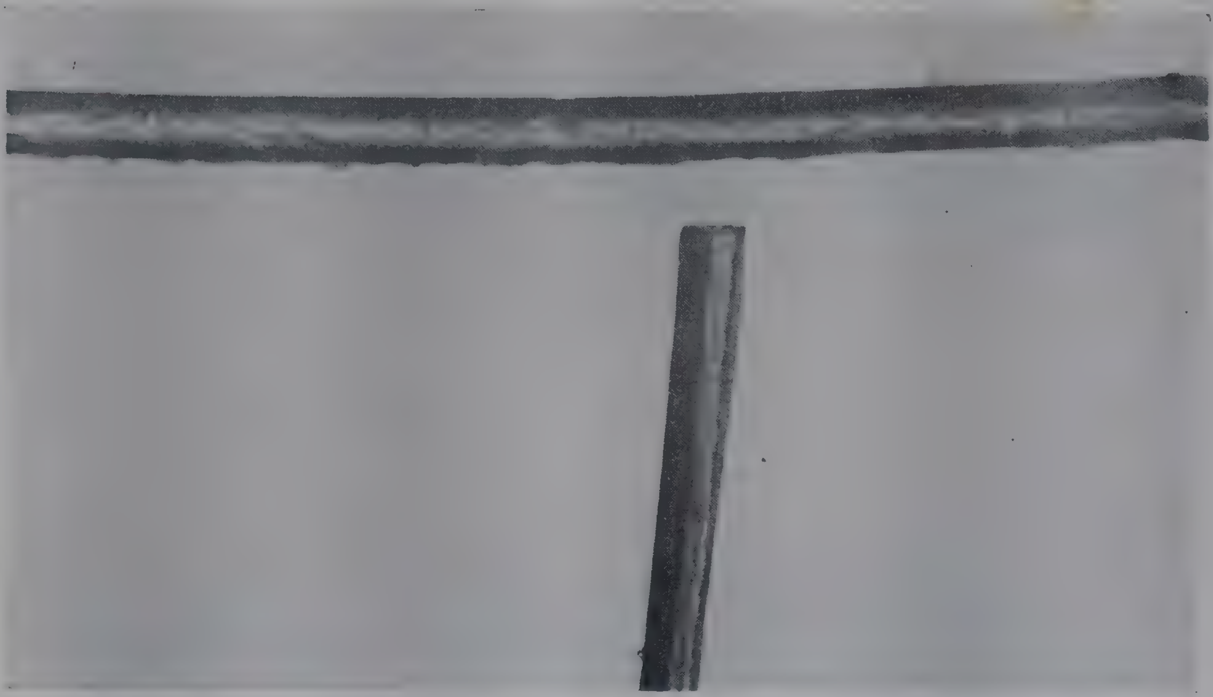


Fig. 679.—Portions of fragments revealing normal hair structure. Case 2. (Courtesy Dr. Israel Castellaños.)

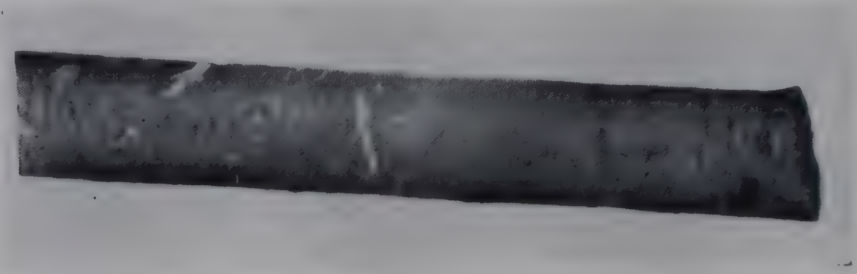
Measurements of diameters of the longest hair, approximately two centimeters long, showed: maximum diameter, 98 microns; average diameter, 56.1 microns; medullary index, 0.25.

The hairs of the "Victim" were light brown according to the Bertillon chromatic scale. Since all of these hairs had been cut, none of them showed the root. These hairs had a definitely imbricated cuticula. On some it was torn, as seen in Fig. 680, *A*, but there were large portions in which the hair was perfectly normal.

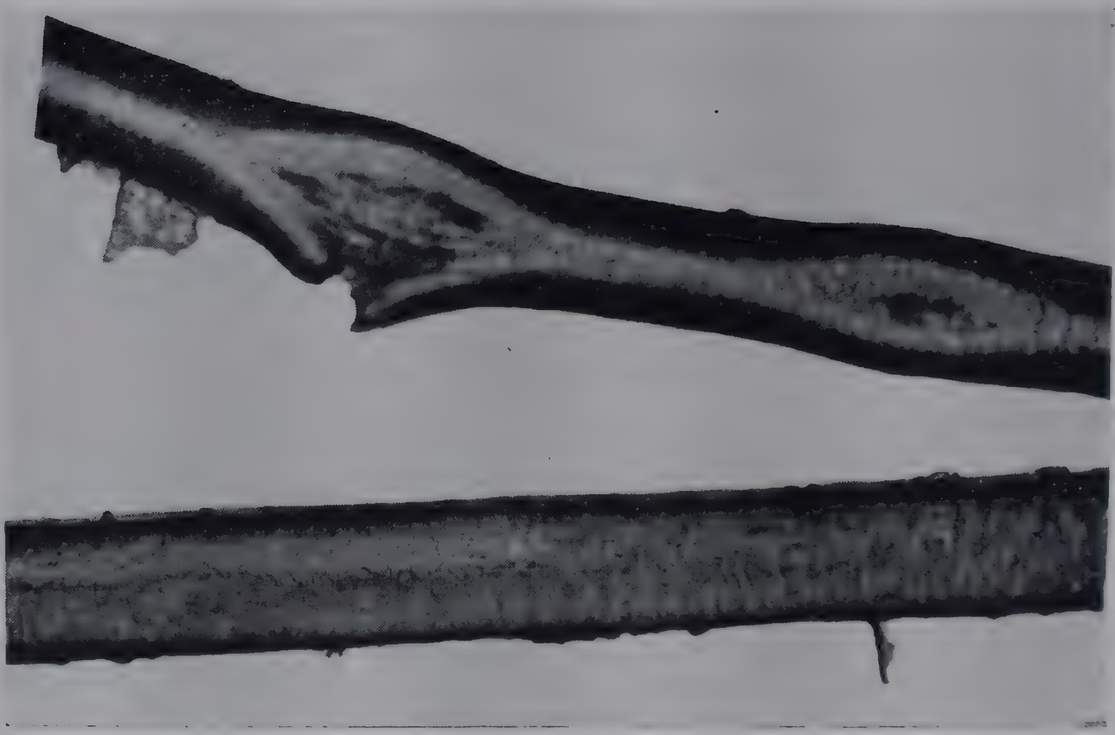
As seen in Figs. 680, *B* and 681, some of the hairs were crushed and broken, thus showing enormous mechanical action. There are large portions, however, in which normal anatomical structure can be observed.

Diameters of the hair coming from the "Victim" were: maximum, 73 microns; minimum, 17 microns; average diameter, 41.5 microns; medullary index 0.23.

The medulla of these hairs was disunited in some regions and invisible in others



A.



B.

Fig. 680.—*A*, Hair showing torn cuticula. *B*, Hair showing green-stick fracture of the stem. Case 2. (Courtesy Dr. Israel Castellaños.)

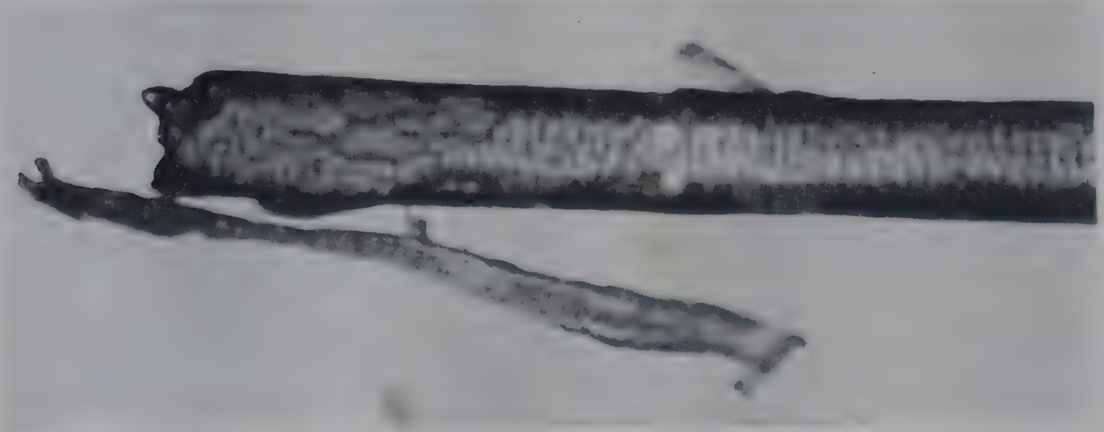


Fig. 681.—Hair which has been crushed and broken, showing mechanical action. (Courtesy Dr. Israel Castellaños.)

In comparing the results of the examinations of hairs marked "Questioned" and "Victim," there can be noted a marked resemblance in the type of the cuticula cells (definitely imbricated) and the pigmentation or coloring. Furthermore, the lesions in the hairs of the "Questioned" are similar to those in the hairs from the "Victim."

Diameters are not the same in the two specimens, but such discrepancies are found when the hairs come from different regions of the body. If hairs from different regions are compared, measurements are of secondary value. Anatomical characteristics, color, pigmentation, the morphologic type of hair, etc., are the decisive factors in making the diagnosis. In Case 2 all details are alike except the measurements.

The fragment of connective tissue was too small to be submitted to all the biological tests. There was not enough to test it serologically to determine whether it was of human or animal origin. It was found with human hairs clinging strongly to it, and the histologic examination does not deny that it is of human origin, so that it can be assumed to be human hair.

Investigations of hairs are always slow but such investigations are extremely important.

CHAPTER XV

TOXICOLOGIC TECHNIC[1]

A complete scientific investigation into the cause of death is a most important civic function and community responsibility. For this investigation, there should be properly trained personnel and well-equipped laboratories.

A toxicologic laboratory as an integral unit of an organization devoted to this subject aids materially in determining the nature and cause of death in many otherwise unsolved cases; helps place the responsibility for industrial poisoning; and aids in ruling out the possibility of foul play in supposedly apparent cases of drowning, carbon monoxide poisoning, and the like.

To illustrate these various possibilities, a few case histories are cited briefly.

(a) A young husband returned home unexpectedly one week-end to find that his wife had been unfaithful to him. Since this was not the first offense, he informed his wife that he intended to leave her and sue for divorce. Hotel accommodations were difficult to find; his wife thus had little trouble in persuading him to remain at home. She succeeded in convincing him that they should have one last celebration before he brought suit for divorce. They made the rounds of the taverns, returning to their home in a somewhat inebriated condition. Shortly after returning to his home, the young man died. Although it was not the primary cause of death, alcoholic intoxication appeared to be present. Autopsy and chemical analysis revealed the presence of large quantities of chloral hydrate. The wife was confronted with this startling information and later confessed that she had poisoned her husband. She gave as her reason his constant determination to obtain a divorce.

(b) An elderly couple were found dead in their hotel room. Autopsy revealed very little that might point to the cause of death, and chemical analysis of the organs was ordered. This analysis failed to reveal anything other than faint traces of cyanide. With this clue, a special examination was made of the lung tissues. Cyanide was revealed in large quantities. The hotel management was confronted with this information. They admitted, under questioning, that the room directly below this couple had been fumigated with cyanide gas, but that the management had failed to order the other apartments vacated, as required by law. They had, by their negligence, contributed directly to the death of these two elderly people.[2]

(c) A young woman was found dead in bed. She was lying in a natural position. All the gas jets in her home were opened. A cursory examination might have suggested carbon monoxide suicide. However, a skilled examiner noted that there were certain peculiarities about the case, and a toxicologic analysis was requested. Examination revealed no evidences of carbon monoxide or other poisons. The husband was questioned and later admitted that he had forced his wife's head onto the pillow and had killed her by asphyxiation. He had then placed her on her back, turned on all the gas jets, and left home for work.[2]

These few cases are cited as an example of the importance of toxicologic examination in death from unknown causes, and to point out the responsibility which the community must assume in the pursuit of justice. These crimes might have remained undetected had no toxicologic investigation been made.

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[1] This chapter has been revised under the direction of Sidney Kaye, formerly Toxicologist to the Research Laboratory of the St. Louis Police Department; at present Toxicologist, Office of Chief Medical Examiner, Commonwealth of Virginia.

[2] A. O. Gettler: Why the Coroner System Has Broken Down, National Municipal Review 13: 10, Oct., 1924.

To date only a few communities in the United States have a medical examiner system to make routine thorough investigations into the cause of death. Such systems are to be found in New York City, Boston, Newark, Nassau County, Cleveland, Los Angeles, Cincinnati, and the States of Maryland, Virginia, Connecticut, and Vermont.

Investigations do not always reveal results favorable to the prosecution as is illustrated in the following case:

A young man was admitted to a hospital suffering from injuries sustained in an automobile accident. Since he was in a serious condition, blood transfusions were indicated and he was given a number of transfusions. Several days later the patient died, and an autopsy was performed. This revealed evidences of nephritis, and a toxicologic analysis was requested. None of the common poisons were found except a faint trace of mercury in the kidneys. All tests for mercury gave specific reactions. These findings were disconcerting because the young man had at first shown signs of recovery, he had the will to live, and had no known enemies; nor did there appear to be any source of error in medication. A thorough investigation of the treatment administered to the patient was ordered. This revealed that the patient had been given two blood plasma transfusions, both containing Merthiolate as a preservative. Merthiolate is sodium ethyl mercuri-thiosalicylate. The presence of Merthiolate in the plasma immediately solved the mystery of the origin of the mercury.

GLOSSARY

Toxicology is the science of poisons and their properties; isolation, purification, identification, and quantitative determination from biologic material; the lethal or fatal dose; the action upon living tissue; and the proper treatment necessary to counteract this action.

A **poison** is any substance which, without acting mechanically, causes a disturbance of body functions, injury, disease, or death, when introduced into a living organism and when absorbed in sufficient amount.

A **potent poison** is usually defined as any substance which is likely to destroy human life or seriously injure health when applied in quantities of less than 5 c.c. or 5 grams.

Minimum lethal dose is the smallest quantity of a poison which, when actually absorbed into the system, has been proved to be fatal.

GENERAL FACTS CONCERNING TOXICOLOGIC ANALYSIS

Toxicologic analysis of biological material must be performed by experts. It should be undertaken only by those with specialized training and experience, since the life and liberty of others may depend upon the results of these investigations. Rough qualitative testing, sometimes attempted by those with little or no experience in forensic chemistry, is most unwise, for at best it consumes irreplaceable material and at worst it may lead to miscarriage of justice.

Qualitative testing of poisons must always be performed with the same care and accuracy used in all micro-quantitative procedures.

Before making an analysis, a careful study of the complete history of the case should be made. This history should include first appearance of symptoms before death; symptoms occurring after taking last food or drink; nature and intensity of such symptoms, whether there was vomiting, deep sleep, tingling of skin, throat, convulsions or twitching of the muscles, delirium, dyspnea, contraction or dilatation of pupils, visual or auditory effects, if any.

Patient's symptoms and other clues may be of great help to the toxicologist in ruling out large groups of poisons which, in view of the history, would not likely be present. Close cooperation with the physician who last saw the patient, and with the pathologist who performed the autopsy and examined the tissues, is solicited.

SYMPTOMS SHOWN BEFORE DEATH

Many diseases cause symptoms which are similar to those caused by poisons. Differentiation between clinical symptoms and symptoms due to poisons is often difficult unless a very careful study is made. It must be kept in mind that unusual symptoms may be produced by a variety of poisons, just as unusual symptoms may occur in disease. The list of Webster[1] below gives the poisons and diseases which produce similar symptoms.

Vomiting, Purging, and Abdominal Pain.—

Poisons: Heavy metal salts such as arsenic, antimony, barium, copper, mercury, zinc, corrosive acids and alkalies, halogens, aconite, cantharides, croton oil, digitalis, gelsemium, phosphorus, phenols, wood alcohol, veratrum, and muscarine

Diseases: Gastritis and enteritis, gastric and duodenal ulcer, cholera, uremia, acidosis, onset of many infectious diseases, early pregnancy, and brain tumor

Convulsions:

Poisons: Ammonium salts, aspidium, brucine, camphor, santonin, aconitine, picrotoxin, cyanides, and strychnine

Diseases: Uremia, eclampsia, tetanus, epilepsy, many acute diseases of the cerebrospinal system, especially meningitis

Coma:

Poisons: Opium, morphine, heroin, barbiturates, chloral, sulphonal, trional, paraldehyde, chloroform, ether, cyanide, carbon monoxide, carbon dioxide, atropine, nicotine, hyoscine, alcohols, phenols, scopolamine

Diseases: Uremia, eclampsia, acidosis, cerebral hemorrhage, cerebral embolism and thrombosis, brain injuries, epilepsy, and other brain diseases

General or Partial Paralysis:

Poisons: Cyanides, carbon monoxide, carbon dioxide, botulism

Diseases: Apoplexy, brain tumor, meningitis

Dilatation of the Pupils:

Poisons: Belladonna and its derivatives, hyoscyamine, stromonium, gelsemium, cocaine, nicotine

Diseases: Those causing optic atrophy, sympathetic irritation, or oculomotor paresis

Contraction of Pupil:

Poisons: Derivatives of opium, physostigmine, pilocarpine, muscarine

Diseases: Tabes and certain other diseases of the central nervous system

Slow Respiration:

Poisons: Opium derivatives, carbon monoxide, hypnotics

Diseases: Uremia, compression of brain from any cause, for example, hemorrhage

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[1] Peterson, F., Haines, W. S., and Webster, R. W.: Legal Medicine and Toxicology, ed. 2, Philadelphia, 1923, W. B. Saunders Co., Vol 2.

Rapid Respiration:
Poisons: Atropine group, cocaine, carbon dioxide
Diseases: Acute respiratory diseases, lesions of medulla, hysteria

Delirium:
Poisons: Atropine group, cocaine, alcohols, camphor, solanine, marihuana
Diseases: Epilepsy, insanity, delerium tremens, organic brain diseases, such as meningitis, encephalitis; nephritis

Dyspnea:
Poisons: Strychnine during the convulsions, cyanide, carbon monoxide, carbon dioxide, easily volatile organic liquids
Diseases: Those of cardiac and respiratory systems, lesions of medulla and vagus

Cyanosis:
Poisons: Nitrobenzene, aniline, acetanilide, opium, chlorates, amyl nitrite
Diseases: Same as dyspnea, prolonged convulsions due to any cause, by producing cardiac embarrassment or dilatation

Many of these clues and leads may prove of great value as ante-mortem symptoms.

ADDITIONAL GENERAL CLUES AND "LEADS" ANTE-MORTEM

Color of the Urine.—

Dark green: phenol, salol, creosote, resorcinol
Red: antipyrine, trional
Brown or black: pyrogallol
Yellow: phenacetin, picric acid. Santonin changes from bright yellow to scarlet upon the addition of sodium hydroxide.

Breath, Odor.—

Phenol: phenolic
Creosote: phenolic
Ether: ethereal
Chloroform: sweetish
Cyanide: bitter almonds
Alcohol: alcoholic
Phosphorus: garlic
Chloral hydrate: pears
Nitrobenzene: shoe polish
And many others

Miscellaneous.—

Saponins: foam persists upon shaking the urine specimen
Quinine, acetanilid, salicylates: depress the heat centers and lower body temperature
Strychnine: may cause tingling of the skin; it increases sensitivity to sound
Salicylates: may cause buzzing of the ear; *quinine* may cause ringing of the ear
Pulse, in acute poisoning: may be quick and feeble due to shock

CLUES AND LEADS FOUND AT AUTOPSY*

Gastrointestinal Tract.—Inflammation, erosion, hemorrhage, ulceration, perforation:

Mineral acids, caustic alkalies, ammonia, heavy metal salts, sodium fluoride, phosphorus, halogens, oxalic acid, aconite, croton oil, cantharides, gelsemium, muscarine, phenols, veratrum, and wood alcohol

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*Based largely on descriptions given by A. O. Gettler in his numerous articles in the literature.

TABLE 216

AUTOPSY CLUES TO COMMONER POISONS

	POISON	ODOR	EXTERNALS	PUPILS	SUPRA-GASTRIC	SUPRA-PULMONIC	STOMACH	BOWEL	LIVER	KIDNEYS	LUNGS	BRAIN	MUSCLE	OTHERS
I	CARBON MONOXIDE		cherry red to dusky violet patches	—	—	rd froth	H2	H2	—	rd H fD	rd C	rd . H2	rd C	omentum, H4
	HYDROGEN CYANIDE	od	asphyxia: red to dusky violet areas, red fading more rapidly than with CO	O O	C4: frothy mucus	frothy mucus	C4: rd. to dark pl.	—	C	—	H:C	—	—	—
II	CHLORAL HYDRATE	—	rashes (chronic poisoning)	OO or oo	C	C	C3	C3	C fD	C fD	edema	C3	fD	—
	CHLOROFORM	od	cyanosis. jaundice (delayed death)	OO	C	C	C	—	fD	fD	C	—	fD	—
	CYANIDES	od	see HCN	OO	rd. N		rd. bu. N							
	ETHYL ALCOHOL	od	jaundice (chronic poisoning)	OO or oo	—	—	rd: C: H	C:H	C	C	C	C	—	—
	FORMALDEHYDE	od	dermatitis (repeated poisoning)	—	C	C	cooked	—	—	—	C: I	—	—	—
	METHYL ALCOHOL	—	cyanosis: red lividity	OO	rd 4	—	rd. 4: C: H	—	C	C	C: edema	N:C4	—	Bladder. C
	PARALDEHYDE	od	—	oo	—	—	I.2	C	C	C	C	C	—	—
	PHENOLS	od	cyanosis: corrosion. bu br. bk pigmentation (delayed death)	—	C: br. N	—	C:I: bu. gy N	C:I	C2	C2	C4: bu. bk. br	—	Pigmented Heart valves	Pigmented aorta
	PHOSPHORUS	od	purple eruptions (Jaundice	—	—	I	fD2: luminous (acute)	N: fD2	yw fD	gy yw. D	edema	D	fD	—
III	BARBITURATES	—	cyanosis urticarial, maculopapular, scarlatiniform rashes: exfoliative dermatitis gangrene of feet	—	C	—	I	—	C4	C	C4:	C4 meninges H2	H2	Uterus. H2
	SALICYLATES	—	cyanosis: swollen jaw: urticarial, erythematous, papular, hemorrhagic rashes: angioneurotic edema	—	—	—	C	C	C	C· H2	C·I	—	—	—
IV	COCAINE GROUP	—	needle marks	OO	—	—	C2	C2	I.4:C2	I.4:C2	I.4:C 2	I. 4	—	—
	CODEINE	—	needle marks: cyanosis	oo or OO	—	—	C	C	C	C	C:I2	C3	—	—
	STRYCHNINE	—	opisthotonos	OO	—	—	C	—	C	C	—	C	—	—
V	MORPHINE GROUP	od?	cyanosis needle marks	oo or OO	—	—	C	C	C	C	C I2	C4	—	—
VI	ARSENIC	—	eruptions of all kinds jaundice keratoses of palms + soles: anxiety	—	I	—	gn mucus. H I	H I	D. H	H fD	C	—	fD	—
	BARIUM	—	—	—	N	—	I: N4	I N4	—	—	—	—	—	—
	CHROMIUM	—	—	OO	N	—	bu gy. N: I	bu gy. N·I colon	D	bu gy D:I	—	bu. gy	—	—
	LEAD	—	expression of anxiety	—	bu gums	—	I.2	gy bk. I.2	—	gy. D:C	—	—	fD	—
	MERCURY	—	—	OO	bu gums N	—	N I	N H colon	—	I 4	—	—	—	—
VIII	BROMIDES	—	follicular pustules inflammatory fungating leg lesions (chronic)	—	—	—	—	—	—	—	—	—	—	—
	FLUORIDES	—	—	—	—	—	rd. bk. N: I. 4	I. 4	—	D	—	—	—	—
IX	BORATES	—	cyanosis eczema	—	—	—	I	I	—	D I	—	—	—	Bladder I
	CHLORATES	—	cyanosis	—	—	—	I 4	I 4	br I	br I 4	—	—	—	Urine bk
X	SULFONAMIDES	—	cyanosis. scarlatiniform, morbilliform, purpuric rashes: erythema multiforme; exfoliative dermatitis erythematous nodules on legs: conjunctivitis	—	—	—	—	—	C4 focal N	D I stones	—	—	—	Bladder stones Thyroid hyperplasia?

ABBREVIATIONS

C	congestion, hyperemia, "injected"
D	degeneration
H	hemorrhage
I	inflammation
N	necrosis, corrosion, ulcers
OO	dilated pupils
oo	contracted pupils
od	odor distinctive, often detectable at autopsy

2	slight
3	moderate
4	severe
—	normal, or no available data

bk	black
br.	brown
bu	blue
gn.	green
gy	gray
pl.	purple
rd.	red
wh.	white
yw.	yellow

Example. I: N4
Inflammation of indefinite severity, and necrosis severe.

DCP, AMS, March 1944

Odor of Gastric Contents.—Volatile substances having specific odors:

Alcohol, benzene, chloroform, cyanide, ether, nicotine, nitrobenzene, opium, phenol, Lysol

Colored Materials in Stomach.—

Grayish-white undissolved material: arsenic oxide, remnants of tablets, etc.
Green or blue coloration: copper salts and dyes added to fluoride and strychnine
Yellow: picric acid, nitric acid
Black: sulphuric acid, oxalic acid
Luminous particles (in the dark): phosphorus
Pink to violet: permanganate
Blue or brown: iodine
Bright red: Mercurochrome

Skin Discoloration.—

Icteric tint: phosphorus, arsenic, alcohol
Cherry red or pink: carbon monoxide, cyanide
Brown, chocolate color: nitrobenzene, chlorates, acetanilid
Blue lividity: carbon dioxide
Dark blue-gray: silver (chronic)
Blue: iodine
Deep brown: bromine
Yellow: nitric acid and picric acid
Bleached: phenols

Gums, discoloration, usually in chronic poisonings.—

Lead, copper, bismuth, mercury, silver

Blood, Color of.—

Chocolate color: nitrobenzene, acetanilid, chlorates
Cherry red or pink: carbon monoxide, cyanide

CLASSIFICATION OF POISONS

Classification of poisons may be made on the basis of (a) their origin, and (b) the effects produced upon the body.

Another classification of poisons is: (1) corrosives, (2) irritants, and (3) neurotics. A better classification is (1) inorganic poisons, (2) gaseous poisons, (3) alkaloidal poisons, (4) nonalkaloidal organic poisons, and (5) food poisons.

The toxicologic investigation of viscera from autopsies where poisons are suspected is a very important part of laboratory procedure. It should not be undertaken by anyone not well-qualified to do this work. It must be remembered that the results of toxicologic examinations are later to be presented in court; one must be prepared to qualify as an expert toxicologist in order that his findings be accepted by a court of justice.

This chapter is not intended to represent a complete treatise on the subject of toxicology; rather it is a working outline. For complete details the reader is referred to standard textbooks on this subject.

There are certain suggestions regarding the choice and handling of specimens for toxicologic analysis which must be considered.

SUGGESTIONS FOR HANDLING OF SPECIMENS

1. **The Quantity of Organs Required.**—The analyst is often hampered on account of lack of material. During the autopsy it is just as easy to procure large samples as small ones. Therefore the pathologist should take out gen-

erous portions of the organs when chemical analysis is to be made. In the case of the stomach, the entire organ, together with all of its contents, should be furnished. This can be accomplished by tying a ligature around the cardiac end and another around the pyloric end. The organ is then removed and placed in the proper receptacle. Portions of the intestines about two feet long of both large and small bowel should be taken. One-fourth of the liver is a proper sample. In the case of the kidneys and other organs, the entire organ should be taken.

2. **Containers.**—Fruit jars are very satisfactory, provided they are scrupulously cleaned. This means chemical and bacteriologic cleanliness. They may be chemically cleaned by washing them thoroughly in a bichromate-acid solution followed by large quantities of distilled water. This may be followed by dry heat sterilizing at 170° C. for two hours. Each organ should be placed in a separate container and should be labeled, giving the date, the place, the name of the deceased, and the name of the autopsy operator. Before each organ is chemically examined, it should be accurately weighed and the amount noted.

3. **Refrigeration.**—If possible the organs should be kept in a refrigerator since those poisons which decompose will do so much more slowly in the cold.

4. **Embalming.**—With a fresh corpse it is much better to secure the samples before the body is embalmed. Certain poisons, such as cyanides, are impossible to analyze after formaldehyde is introduced into the body. If the body has been embalmed, determine, if possible, whether the poison found was in the embalming fluid. Some embalming fluids may contain poisons. Formaldehyde interferes with the identification of methyl alcohol, alkaloids, etc., in addition to hardening the tissue and making poison extraction extremely difficult.

5. **Preservatives.**—No preservative should be added to the organs. Blood for alcohol determination is an exception. Here one adds sodium fluoride, 200 mg. for each ounce of blood.

6. **Speed in Transmission.**—Since several of the poisons are destroyed after a time, it is best to send the organs as soon as possible. However, some poisons, such as arsenic and mercury, are not affected by standing in contact with tissues and these may be found many years after burial, if they were originally present.

7. **Precautions Against Tampering With Material for Analysis.**—In analyses, reports of which may be used in court, it is absolutely necessary to show that no one but the pathologist and analyst, or some other responsible person, had access to the material. The parts should be transported from the autopsy room to the laboratory by the pathologist himself, or by some responsible person, receiving a receipt for them. The receptacles containing the specimens should be sealed and placed under lock and key pending investigation, and during the tests.

It will be of material assistance to the chemical expert to have a brief description of the results of the autopsy. Often the macroscopic findings in the body will give the toxicologist a clue as to the particular poison to be looked for.

In the case of exhumed bodies which have already undergone decomposition, it is well to remember that poisons from the coffin may contaminate the body. It is then necessary to make a chemical analysis of the material from which the coffin is made. In cases where the body has been buried for a long time, and where the coffin has undergone decay, it is also necessary to take parts of the surrounding earth for examination.

For a complete chemical analysis the following suggestions are offered for packing and transportation of viscera:

Vessel No. 1: Stomach opened up with its contents.
Vessel No. 2: Large intestine opened up with contents.
Vessel No. 3: Small intestine opened up with contents.
Vessel No. 4: Liver, kidneys, and spleen. If possible, each kidney should be put in a separate vessel.
Vessel No. 5: Urine.

Where volatile poisons are suspected:

Vessel No. 6: Brain and organs of the chest.

Where it is thought a bacteriologic food poisoning is present:

Vessel No. 7: A doubly tied-off piece of small and large intestine.
Vessel No. 8: Sample of blood.
Vessel No. 9: Gall bladder tied off.

If there is a suggestion of the kind of poisoning present:

Vessel No. 10: Large amount of blood.

In carbon monoxide poisoning, we should examine preferably blood from the heart and the sinuses of the head.

There are a number of points relative to the chemical examination of suspected material in medicolegal cases that must be constantly borne in mind. Prior to necropsy, most cases to be investigated for evidence of poisoning will fall into one of three general categories (Maldeis[1]): category I, cases in which the cause of death has already been established and the nature of the poison determined; category II, where the evidence is such that poisoning is regarded as the probable cause of death; category III, cases in which there is so little information pertaining to the cause of death that poisoning is regarded as possible rather than probable. From the standpoint of the law it is not enough to establish the fact that a certain poison was present in the body of the decedent, although such information might be considered adequate in some instances from a medical standpoint. Further, it should be remembered that the identification of the kind of poison and the demonstration that death was due to that poison is only half of the chain of evidence essential to the administration of justice. The law is equally concerned with the acquisition of evidence relating to the identity of the person or persons through whose negligent or intentional act the poisoning occurred. The idea of probable poisoning may exist prior to the performance of the necropsy and this probability may rest on the unexpected and unobserved occurrence of death together with the finding of a suicide note or the finding of a poison that had been accessible either to the decedent or to someone who might have profited by his death. In suicidal poisoning the toxicologic search may be guided by information obtained as a result of searching the premises and belongings of the decedent. In the third category, cases come to autopsy with no positive evidence relating to the cause of death but in which poisoning is regarded as a possibility. Often the problem is settled by the finding at autopsy of some disease process which constitutes an adequate explanation of death. In some cases, however, the autopsy may fail to disclose the cause of death, and search for poisoning must be made. This search varies according to the exigencies of the situation and according to the facilities of the investigator for the conduct of toxicologic studies.

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[1] Am. J. Clin. Path. 13: 165, 1943.

In addition to the material already catalogued for routine collection, Maldeis advises the following material should also be collected: all of the bladder urine; half of each of three vertebral bodies; and about 5 grams of hair.

Emphasis must be placed upon certain factors in toxicologic cases relative to reception of the specimens from the referring person if the toxicologist is not present at the autopsy. The exact time and date of arrival should be noted and recorded. The name of the person receiving the specimen should be recorded. The number of containers and the labelling of each should be recorded. The type of container and whether each is wrapped or sealed should also be recorded. Laboratory numbers should be given to all specimens immediately. The entire material should, after its reception in the consulting laboratory, be taken to the toxicologic laboratory and promptly placed under lock and key.

During the performance of the toxicologic investigation, all visitors should be excluded from the laboratory. The toxicologist must be able to certify in court that no one had access to the material while in his custody. In a case in St. Louis, a toxicologist was placed in an embarrassing position by being unable to complete his analysis for the reason that a porter in his laboratory had washed up the final residue of his extraction and also washed up the container with the original specimen.

It is interesting to note the problems which beset a toxicologist (Wittaus).

1. Was death due to poison?
2. Is there any chance for simulation?
3. Could there be poisoning without demonstration of the poison?
4. Which poison caused the symptoms before death?
5. Are the chemical substances recovered really the causative poison?
6. When extremely small amount of the poison is found, could this poisonous substance of such small amount cause death?
7. Could this substance have caused death?
8. Was the poison taken in sufficiently large quantity to cause death?
9. Was the poisoning suicide, accident, or murder?
10. Could the poisonous substance found have been used as a medicinal agent?
11. When and how was the poison taken?

12. Was the chemical substance found really taken by the subject intra vitam? Could another origin have existed?

13. Is the meat of poisoned animals poisonous for man?

14. Can one determine whether or not death by poison occurred in burned bodies?

TABLE 217.—MATERIAL BEST SUITED FOR ANALYSIS

SPECIMEN	MINIMUM AMOUNT NEEDED	POISON FOR WHICH BEST SUITED
Urine	All available	For most types of poisoning
Stomach	All available	For examinations in which poison is alleged to have been taken by mouth within a short time of death
Intestinal contents	All available	For poisons taken by mouth within one or two days before death
Blood	Approximately 100 c.c.	Barbiturates, cyanide, methemoglobin, carbon monoxide, sulfonamides, bromides, for the drowning test, alcohol determinations
Brain	500 grams	Volatile poisons, barbiturates, alkaloids, and acute alcoholism
Liver	300 grams	Heavy metals, barbiturates, fluorides, oxalate, sulphonal
Kidney	One kidney	Metals, especially mercury, sulfonamides
Bone	200 grams	Lead, arsenic, radium (especially chronic), and other radio-active substances
Lung	One lung	For inhaled poisons as proof of entry
Hair and fingernails	10 grams	Chronic arsenic poisoning
Muscle	200 grams	In most acute poisoning and when internal organs are badly putrified

ANTIDOTES[1]

An antidote is any agent which will remove or prevent the absorption of a poison. There are three types of antidotes: mechanical, chemical, and physiologic.

Mechanical antidote: Removes a poison or prevents its absorption

Chemical antidote: Acts chemically to form a nontoxic compound

Poisons are rendered nontoxic by formation of an insoluble compound, formation of a soluble nontoxic compound, or by oxidizing the poison

Physiologic: One whose action on the body is opposite to that of the poison

Symptoms of Poisons

Absorbed poisons quickly pass from the blood stream into the tissues of the various organs. The poison interferes with the normal functions of the organs and causes abnormal or pathologic symptoms. Poison is indicated if an individual suffers intense pain, becomes delirious, sinks into a stupor, has convulsions, or vomits freely, etc., after taking a drug or food, provided the symptoms are not caused by disease. Symptoms may vary with the amount of poison taken and absorbed, and with the length of time it remains in the body. Most poisons exhibit three stages of poisoning. Treatment varies with the degree or stage of poisoning.

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[1] Compiled from the literature.

First Degree Poisoning

First degree poisoning is of brief duration. If the patient is observed in this stage, much of the poison is still in the stomach, and mechanical and chemical antidotes are employed.

Second Degree Poisoning

Second degree poisoning is the period in which marked symptoms are produced. These symptoms indicate the true nature of the poisoning. Physiologic antidotes are first administered in small and cautious amounts to counteract the original poison. Mechanical and chemical antidotes follow, to remove any residual poison still in the stomach.

Third Degree Poisoning

Third degree poisoning is the stage during which the poison produces the full effect upon the body. The vital organs are depressed, often to the point where function ceases and death follows. Physiologic antidotes and measures are used.

EMERGENCY TREATMENT FOR POISONING[1]

General Rules

1. Immediate action is necessary.

2. Call a physician.

3. Proceed at once to induce vomiting repeatedly except in those cases where poisoning is due to *strong acids* or to *strong alkalies.* The most commonly used *emetic* is that prepared by stirring one teaspoonful of powdered mustard in sufficient warm water to make a creamy paste. If mustard is not available a solution of 0.006 gram of potassium antimony tartrate (tartar emetic) in 25 c.c. of water can be administered; or warm salt water or even ordinary soap suds may be used; or zinc sulphate, 30 to 60 grains, in water.

4. Where the specific antidote is not indicated or where the nature of the poison is not known, administer the universal antidote described below.

5. In cases of poisoning by corrosive substances, the treatments described above may be followed by demulcent drinks to soothe the injured tissues. A convenient *demulcent* is made by dissolving 3 to 5 grams of dried egg albumin in a glass of cold water. The white of an egg or a glass of milk, or both, also are good demulcents. If none of these are available, administer a tablespoonful of mineral oil, or olive oil. Cream, flour, oatmeal gruel, or barley gruel are also fair demulcents.

6. Stimulate, if indicated, with appropriate agents; e.g., cardiac stimulation, respiratory stimulation; combat shock or collapse.

7. As a general rule, the patient should be given a purgative.

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[1] Laboratory Emergency Chart, Fisher Scientific Co., and other sources.

Universal Antidote

Pulverized charcoal	2 parts
Magnesium oxide	1 part
Tannic acid	1 part

Keep dry until ready to use. Dose: one heaping teaspoonful in a small glass of warm water.

TREATMENT FOR SPECIFIC POISONS

(Swallowed, unless otherwise specified)

Acetone.—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer universal antidote.
3. Keep patient awake. Administer strong coffee, etc.

Acetylene.—

See ''Carbon Monoxide Treatment.''

Acids, Mineral.—

1. Do **NOT** induce vomiting. Do **NOT** use stomach pump!
2. Administer magnesium oxide, milk of magnesia, or lime water immediately. Repeat the dose at short intervals. Administer milk or white of egg, or 5 grams of egg albumin in cold water, or other demulcants. Do **NOT** give carbonates.
3. After the acid appears to be neutralized, administer a tablespoonful of mineral oil until 4 doses, 15 minutes apart, have been given.
4. Combat collapse and shock by stimulation.

Alcohols, Methyl, Amyl, Etc.—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer milk or white of egg, or 5 grams of egg albumin in cold water.
3. Combat collapse: Keep patient warm, give strong coffee, aromatic spirits of ammonia, or digitalis or strychnine.
4. If patient is not breathing, or if he is breathing with difficulty, apply artificial respiration at once. AFTER TREATMENT: Plenty of fresh air, ½ gram sodium bicarbonate in water every 2 or 3 hours, cathartic.

Aldehydes (Formaldehyde, etc.).—

1. Artificial respiration, if necessary.
2. When patient is conscious, administer repeated doses of 1 to 2 c.c. of 1 per cent ammonium hydroxide solution. Follow by alkaline mineral water or 5 per cent solution of sodium bicarbonate.
3. Administer white of an egg, milk, or barley water, or 5 grams of egg albumin in cold water.
4. Combat collapse: Keep patient warm, give strong coffee, aromatic spirits of ammonia.

Alkalies, Caustic.—

1. Do **NOT** induce vomiting, and do **NOT** use stomach pump.
2. Administer 5 per cent acetic acid or vinegar or lemon or grapefruit juice until alkali appears to be neutralized.
3. Administer white of egg, milk, or 5 grams of egg albumin in cold water, or other demulcents.

Ammonia Gas.—

See ''Corrosive Gases.''

Aniline and Derivatives.—

1. Artificial respiration, if necessary.
2. When patient is conscious, induce vomiting. See ''Poisoning, General Rules.''
3. Administer 5 per cent solution of acetic acid.
4. Combat collapse: Strychnine, atropine, or digitalis by hypodermic injection as a stimulant.

Antimony Compounds.—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer strong tea or 2 grams of tannic acid in a glass of warm water.
3. Administer milk or white of an egg or 5 grams of egg albumin in cold water, or other demulcents.
4. British anti-lewisite (BAL) intramuscularly.

Arsenic Compounds (White Arsenic, Arsenates, Arsenites).—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer a solution of 4 grams of sodium thiosulphate in 450 c.c. of water.
3. Administer a tablespoonful of milk of magnesia or a glass of milk or the white of an egg or 5 grams of egg albumin in cold water.
4. Combat collapse: Strychnine, atropine, or digitalis as a stimulant by hypodermic injection.
5. Follow treatment with one ounce of castor oil.
6. British anti-lewisite (BAL) intramuscularly.

Barbital Group (Amytal, Pentothal, Seconal, Nembutal, Phenobarbital, etc.).—

1. Irrigate stomach with tannic acid solution followed by water.
2. Give stimulants, strychnine hypodermically; or benzedrine sulphate ⅔ grain intravenously, then ⅓ grain every hour; the physician must keep careful watch over the patient.
3. High colonic irrigation. Diuretics, catheterization of bladder, and artificial respiration with oxygen when necessary.

Barium Compounds.—

1. Induce vomiting. Mustard emetics or gastric lavage followed by dose of Epsom salts.
2. Administer a solution of 4 grams of sodium thiosulphate in 450 c.c. of water.
3. Administer milk, white of an egg, or 5 grams of egg albumin in cold water.
4. Stimulant by hypodermic if necessary.

Bromine Solutions.—

1. Induce vomiting by emetics.
2. Administer a solution of starch and 5 per cent sodium bicarbonate.
3. Administer milk or white of egg or 5 grams of egg albumin in cold water, or other demulcents.
4. Morphine hypodermically for pain.

Bromine Gas.—

See ''Corrosive Gases.''

Carbolic Acid (Phenol).—

1. Administer *immediately* 4 ounces of ethyl alcohol in 4 ounces of water, or one-half pint of whisky or gin.
2. Induce vomiting. See ''Poisoning, General Rules.''
3. Administer milk, or 5 grams of egg albumin in cold water, or mineral oil.
4. Morphine by hypodermic injection for pain. Stimulants if necessary.

Cantharides.—

1. Emetic or stomach tube, followed by demulcents.
2. Drink plenty of water.
3. External heat application.
4. Morphine ¼ grain by hypodermic injection to relieve pain

Carbon Monoxide, Carbon Dioxide. Acetylene, Ethylene, Hydrogen Sulphide, Illuminating Gas, Nitrous Oxide, Phosgene.—

If Inhaled.—

1. Remove patient to fresh warm air immediately.

2. Apply artificial respiration, if necessary, for 4 hours, during which time administer oxygen if available.

3. Keep patient warm, using blankets, jars of warm water against the body, etc. Stimulants.

Chloral Hydrate (''Knock-Out Drops'').—

1. Emetic. Or irrigate stomach with large amounts of warm water.

2. Give strong coffee or caffeine orally, or strychnine sulphate 1/60 to 1/20 grain by hypodermic injection as stimulants.

3. Keep body warm.

Chlorinated Hydrocarbons.—

1. Irrigate stomach.
2. Saline cathartic.
3. Artificial respiration if necessary.
4. Calcium gluconate, intravenous or intramuscular.

Chlorine Solution.—

1. Administer 2 c.c. of ammonium hydroxide in 100 c.c. of water.
2. Induce vomiting. See ''Poisoning, General Rules.''
3. Administer milk or white of egg or 5 grams of egg albumin in cold water.
4. Administer 4 grams of sodium thiosulphate in 450 c.c. water.
5. Stimulants if necessary.

Chlorine Gas. —

See ''Corrosive Gases.''

Chromium Compounds.—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer milk or white of egg or 5 grams of egg albumin in cold water.
3. Stimulants if necessary, morphine by hypodermic injection, for pain.

Copper Compounds.—

1. Induce vomiting. See ''Poisoning, General Rules.''
2. Administer 100 c.c. of 10 per cent monobasic sodium phosphate solution.
3. Stimulants if necessary, morphine for pain, by hypodermic injection.

Corrosive Gases.—

Ammonia, Bromine, Chlorine, Hydrochloric Acid, Hydrofluoric Acid, Nitric Acid.

If Inhaled.—

1. Remove patient to fresh warm air immediately.

2. Apply artificial respiration, if necessary, for 4 hours.

3. If patient is conscious, permit the inhalation of vapors of dilute acetic acid if ammonia is active agent; inhalation of vapors of dilute ammonium hydroxide is helpful if bromine, chlorine, hydrochloric acid, or nitric acid fumes are the active agents.

4. Stimulants if necessary, hypodermic injections of morphine for pain.

Cyanides. SPEED IS ESSENTIAL.—

(a) If Swallowed.—

1. Artificial respiration, if patient is unconscious or breathing with difficulty.

2. Induce vomiting. See ''Poisoning, General Rules.''

3. Administer 250 c.c. of water containing 10 c.c. of 3 per cent hydrogen peroxide, or 250 c.c. of freshly prepared solution containing 5 per cent ferrous sulphate and 5 per cent sodium bicarbonate. Inhalation of vapors of *amyl nitrite*.

4. Combat collapse.

5. Inject intravenously *p*-aminopropiophenone followed by intravenous injection of sodium thiosulphate. Equally effective is sodium nitrite followed by sodium thiosulphate, both given intravenously. These antidoes are available in ampule form from many biological houses.

(b) If Inhaled.—

See "Carbon Monoxide Treatment."

Digitalis.—

1. Tannic acid solution, 60 grains to pint of water, and irrigate stomach or administer emetics.
2. Recumbent position for several days.
3. Artificial respiration if necessary.
4. External heat.

Ergot.—

1. Suspension of powdered charcoal in water, then irrigate stomach.
2. Give purgatives: calomel or castor oil.
3. Stimulants: hot strong coffee, caffeine with sodium benzoate (hypodermic). Amyl nitrite by inhalation.

Ethylene.—

See "Carbon Monoxide Treatment."

Fluorides.—

1. Induce vomiting.
2. Administer milk or white of egg or 5 grams of egg albumin in cold water or lime water, then irrigate stomach.
3. 20 c.c. calcium gluconate solution intravenously.
4. Combat collapse: stimulants, artificial respiration if necessary.

Fluoracetate, Monosodium (1080).—

1. Keep patient quiet.
2. Induce vomiting with emetics.
3. Induce purging with cathartics.
4. Sedation for short time.
5. Complete rest (for cardiac symptoms).

Formaldehyde.—

See "Aldehydes."

Hydrochloric Acid Gas.—

See "Corrosive Gases."

Hydrogen Sulphide Gas.—

See "Carbon Monoxide Treatment."

Illuminating Gas.—

See "Carbon Monoxide Treatment."

Iodine.—

1. Administer thin paste of soluble starch in water, or milk, or egg albumin.
2. Induce vomiting. See "Poisoning, General Rules."
3. Combat collapse with stimulants: black coffee, aromatic spirits of ammonia. Morphine by injection for pain.

Lead Compounds.—

1. Induce vomiting. See "Poisoning, General Rules."
2. Administer 4 grams of sodium thiosulphate in 450 c.c. water or 10 per cent magnesium sulphate solution.
3. Administer milk or white of egg or 5 grams of egg albumin in cold water, or other demulcents.
4. Combat collapse by stimulation by hypodermics.

Methanol.—

See "Alcohols, Methyl, Amyl, etc."

Mercury Compounds.—

1. Induce vomiting. See "Poisoning, General Rules."
2. Administer milk or white of egg or 5 grams of egg albumin in cold water.
3. Administer 4 grams of sodium thiosulphate in 450 c.c. of water.

4. Combat collapse: external heat, morphine for pain.
5. British anti-lewisite (BAL) intramuscularly.

Nitrobenzene and Compounds.—

1. Artificial respiration, if necessary.
2. Induce vomiting, with emetics.
3. Administer 100 c.c. of 3 per cent solution of acetic acid, followed by large quantities of water.
4. Combat collapse, with stimulants.

Nitric Acid Fumes.—

See "Corrosive Gases."

Oxalic Acid and Oxalates.—

1. Induce vomiting, using mustard emetic.
2. Administer milk, magnesium oxide paste, or 5 grams of egg albumin in cold water.
3. Combat collapse by administering black coffee, caffeine with sodium benzoate.

Permanganates.—

1. Induce vomiting. See "Poisoning, General Rules."
2. Administer milk or white of egg or 5 grams of egg albumin in cold water, or other demulcents.
3. Administer 5 c.c. of 3 per cent hydrogen peroxide in 100 c.c. of water slightly acidified with acetic acid.
4. Combat collapse by stimulation.

Petroleum Products (Kerosene, Benzine, Gasoline).—

1. Induce vomiting with emetics.
2. Symptomatic stimulation.
3. Artificial respiration if indicated.

Phenol.—

See "Carbolic Acid."

Phosgene.—

See "Carbon Monoxide Treatment."

Phosphorus and Compounds.—

1. Induce vomiting, with emetics.
2. Administer 2 grams of copper sulphate in 100 c.c. water.
3. Administer large quantities of water and 5 to 10 c.c. of oxidized turpentine (turpentine which has had long exposure to the air). Do **NOT** give milk or white of egg.
4. Administer 15 grams of magnesium sulphate in 100 c.c. water.
5. Morphine hypodermically for pain.

Pyridine and Compounds.—

1. Induce vomiting, with emetics.
2. Administer 2 grams of tannic acid in 50 c.c. of water, or universal antidote (above).
3. Combat collapse by artificial respiration and stimulants as indicated.

Silver Compounds.—

1. Induce vomiting, with emetics.
2. Administer 100 c.c. of 25 per cent sodium chloride solution.
3. Administer milk or white of egg or 5 grams of egg albumin in cold water, or other demulcents.

Sulfonamides.—

1. Thoroughly irrigate the stomach.
2. Follow by cathartic, intake of large quantities of water, and catheter drainage.

Tin Compounds.—

1. Induce vomiting, with emetics.
2. Administer 4 ounces of milk of magnesia or mineral oil.
3. Stimulants if indicated.

Zinc Compounds.—

1. Induce vomiting. See "Poisoning, General Rules."
2. Administer large quantities of 5 per cent sodium bicarbonate solution.
3. Administer milk or white of egg or 5 grams of egg albumin in cold water.
4. Combat collapse with stimulants.

TOXICOLOGIC METHODS

Actual testing for poisons presents no great difficulty if the poison is present in moderate amounts, but considerable skill and experience are required to separate poisons from extraneous materials and to purify them before specific tests are applied for their identification.

It is impractical to describe all of the different separation methods used to isolate poisons since they vary with the nature of the material from which they are extracted, and with the poison. Nor is it the intent of this chapter to cover thoroughly all phases of the many poisons. Only those poisons most commonly encountered have been selected for discussion.

For practical purposes, the general scheme of Stas-Otto[1] is used for isolation of many of the common poisons. These are divided into several groups. Isolation may be performed serially in many cases.

Group I. *Volatile Distillation:* Alcohols, cyanides, phosphorus, ether, chloral hydrate, chloroform, phenols, acetone, aldehydes, camphor, aniline, toluene, benzene and derivatives, carbon disulphide, pyridine, methyl bromide, etc.

Group II. *Acid-Ether Extraction:* Barbiturates and derivatives, salicylates, acetamides, oxalic acid, benzoic acid, caffeine, cantharides, acetanilid, phenacetin, picrotoxin, antipyrine, sulphonal group, etc.

Group III. *Alkaline-Ether Extraction:* Alkaloids such as strychnine, morphine, quinine, atropine, cocaine, codeine, brucine, aconitine, emetine, nicotine, yohimbine, pilocarpine, etc.

Group IV. *Heavy Metals:* Arsenic, mercury, antimony, bismuth, selenium, lead, cadmium, zinc, thallium, etc.

Group V. *Poisonous Gases:* Carbon monoxide, hydrogen cyanide, hydrogen sulphide, oxides of nitrogen, etc.

Group VI. *Special Methods.*

These groups are usually adequate for isolation of many of the common poisons. It must be remembered, however, that even some of these require special treatment (aniline, phosphorus, ether, chloroform, morphine, cyanides), while others (fluorides, carbon monoxide, halogens, toxalbumins, benzedrine) require specific procedures.

THE ISOLATION AND IDENTIFICATION OF POISONS

The first step in the identification of poisons is separation into characteristic groups. When the group to which the poison belongs is known, special tests are carried out for the actual identification of specific substances within the group. Classification of these poisons in groups is given above.

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[1] Stas: Bull. de L'Académie de Médicine de Belgique 6: 304, 1851, Brussels. Otto: Detection of Poisons, p. 160.

GROUP I. ISOLATION OF VOLATILE POISONS

Volatile poisons must first be separated from body tissues or fluids by steam distillation. Set up the apparatus as shown in Fig. 682. Place a definite weight of finely ground material in a Florence flask of appropriate size and add two or three times its volume of distilled water. Acidify with several c.c. of saturated solution of tartaric acid and add a little mineral oil to reduce any foam which may develop. Connect the flask to the steam distillation apparatus, start the distillation, and collect an exact volume of distillate in a small container packed with ice.

Now test the distillate for the presence of various volatile poisons. Unless otherwise specified, 10 to 20 c.c. of distillate usually suffice for each test.

Several procedures used in distillation for identifying certain poisons vary slightly from this method. These procedures are described in the specific method of testing for these volatile poisons.

Alcohol is discussed first since it is the most common volatile poison.

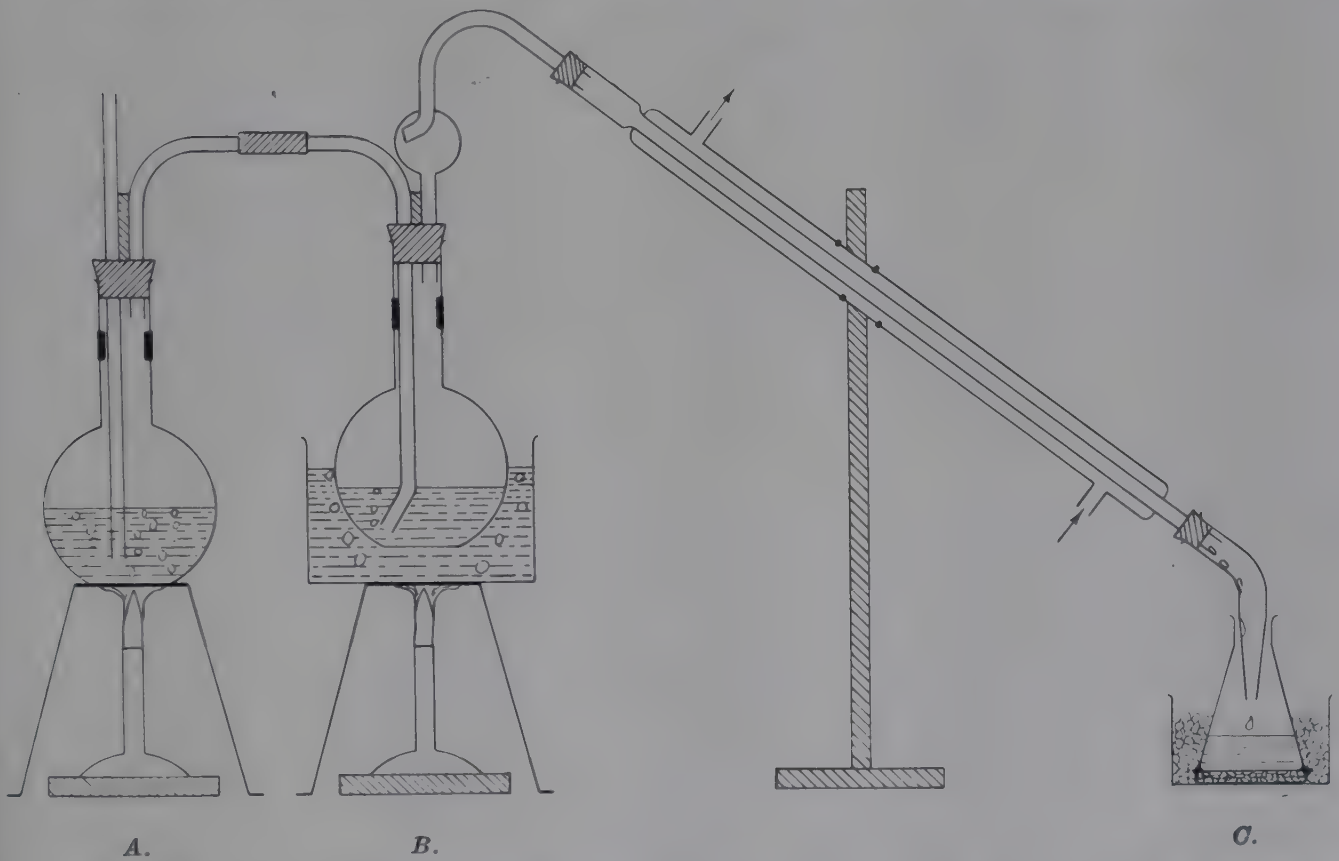

Fig. 682.—Steam distillation apparatus: *A*, distilled water; *B*, specimen in the Florence flask placed in a container partially filled with tap water; *C*, distillate caught in flask submerged in cracked ice.

THE QUANTITATIVE DETECTION OF ETHYL ALCOHOL IN HUMAN TISSUES

A number of methods have been described in the literature for the detection of ethyl alcohol in the human tissues. The first method, described by Subbotin,[1] was based on the oxidation of the alcohol to acetic acid and titration of the latter. Strassman[2] used another method, namely, a colorimetric comparison of solutions of alcohol of definite strengths, using Bodlander's principle of the change of the dichromate from orange to green. Benedict and Norris[1] completely oxidized all the alcohol by an excess but measured amount of a standard solution of dichromate-sulphuric acid. They then reduced the chromic acid that was left by an excess but known amount of a standard solution of ferrous ammonium sulphate. Next they titrated the solution of ferrous ammonium sulphate that was in excess by a standard solution of potassium permanganate until a pink end point was obtained. Another method which depends on the oxidation of the alcohol to acetaldehyde and then ascertaining the amount of the latter colorimetrically by reduced fuchsin has been described.

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[1] Ztschr. f. biol. Chem. 7: 361, 1871.

[2] Pfuger's Arch. f. d. ges. Physiol. 49: 315, 1891.

A fourth method depends on the formation and estimation of iodine derivatives of the ethyl alcohol. A fifth method depends on physical properties; for instance, Sidersky recommended the determination of alcohol by the increased miscibility of ether and water in the presence of alcohol.

DETERMINATION OF ALCOHOL IN BODY TISSUES OR BLOOD

Method of Choice

Method of Sidney Kaye and Abraham Stolman

To determine the presence or absence of alcohol in body tissues, select brain or liver for the tests. If alcohol is found in these tissues, one is able to draw certain conclusions as to the degree of intoxication present. Many methods for determination of alcohol in tissue have been described in the literature. The method recommended by the United States Army, if slightly modified, has proved very satisfactory. Recovery yields with this method are good. The method is simple, and requires no special equipment. It can be completed in very little time.

Principle.—

A steam distillate is collected from a unit weight of brain. A modified Nicloux reaction is performed upon a small aliquot of distillate. By adhering closely to the technic, accuracy of recovery will reach 95 per cent.

Various brain-distillate ratios were attempted experimentally. It was found that by distilling 200 grams of brain and collecting 400 c.c. of distillate maximum recovery of alcohol resulted. It was also found that an ice pack surrounding the distillate during steam distillation is essential if loss of alcohol is to be prevented. Eight c.c. of distillate were found to be ideal for the modified Nicloux procedure.

The **Nicloux procedure** is based on the reduction of potassium dichromate by alcohol. The resultant change in color is compared with permanent color standards representing various concentrations of alcohol.

Since the colors of artificial standards are not quite true in all cases, actual conditions of the test are simulated by using an alcohol-free distillate to which are added standard quantities of alcohol. The Nicloux reaction is then made on these standards.

With the Nicloux reaction, objectionable foaming is produced during aspiration. This may be sucked up into the color reaction tube. If an empty safety tube is interposed between the distillate and the color reagent during suction, this objectionable feature of the reaction will be eliminated. Instead of interposing the empty tube, 0.5 c.c. of castor oil with 0.5 c.c. of oil of petrolatum may be added to the tube containing the test to prevent foaming.

Interference in the reaction by other reducing substances, such as methyl alcohol, acetone, and others, is possible, but these reducing substances may be detected by other qualitative tests on the original distillate. Gettler's procedure is particularly good for the detection of methyl alcohol. Adding Scott-Wilson reagent will inhibit possible interference by acetone in the reaction.

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[1] J. Am. Chem. Soc. 20: 293, 1898.

Reagents.—

Saturated Solution of Tartaric Acid.—

Dissolve 20 grams of tartaric acid in 100 c.c. of distilled water.

Anstie's Reagent.—

Dissolve 3.70 grams of potassium dichromate in 150 c.c. distilled water.

Add 280.0 c.c. of concentrated sulphuric acid.

Dilute to 500 c.c. with distilled water.

Allow to stand 24 hours before using.

Scott-Wilson Reagent.—

Dissolve 5 gm. mercuric cyanide, c.p., in 300 c.c. distilled water.

Dissolve 90 gm. sodium hydroxide, c.p., in 300 c.c. distilled water.

Dissolve 1.5 gm. silver nitrate, c.p., in 200 c.c. distilled water.

When the sodium hydroxide solution is cool, add it to the mercuric cyanide solution.

Mix thoroughly. Then add the silver nitrate solution and again mix thoroughly.

If the solution becomes cloudy, or a precepitate forms on standing, filter.

Note: This solution is poisonous. *Do not pipette!*

Stock Alcohol Solution (2%).—

Place 50 c.c. of distilled water in a 100 c.c. volumetric flask.

Add 2.53 c.c. of absolute alcohol (200 proof), using an accurate pipette, and holding the pipette near the surface of the water.

Dilute to 100 c.c. with distilled water.

Standard Alcohol Solutions (Permanent Standards).—

Have on hand alcohol-free tissue distillate.

Arrange a series of 8 test tubes in a row and place 10 c.c. of the alcohol-free distillate in each. Then add to this the given amount of alcohol and distilled water mixture as indicated in Table 218.

TABLE 218

STOCK ALCOHOL SOLUTION	DISTILLED WATER	CORRESPONDS TO ALCOHOL IN SPECIMEN, MG. PER 100 GRAMS
0.0	1.0	negative
0.1	0.9	0.05
0.2	0.8	0.10
0.3	0.7	0.15
0.4	0.6	0.20
0.5	0.5	0.25
0.6	0.4	0.30
0.7	0.3	0.35

Carry out the Nicloux reaction as described below.

Dilute the contents of each tube to 10 c.c. with Anstie's reagent.

Transfer to clean, approximately matched ampules. These ampules may be prepared by drawing out approximately matched test tubes.

Label and store in the dark. Standards prepared in this manner and kept away from light when not in use will be stable for at least one year.

Technic.—

Mix 200 gm. finely ground brain or liver with 300 c.c. distilled water in a Florence flask which can be connected to a distillation apparatus. (See Fig. 682.)

Acidulate with several c.c. of saturated solution of tartaric acid.

Add approximately 5 c.c. of oil of petrolatum to reduce foaming.

Connect with the steam distillation apparatus. (See Fig. 682.)

The distillate is collected in an Erlenmeyer flask packed in ice.

The adapter which enters the Erlenmeyer flask is elongated so that it lies below the level of the ice pack.

The receiving vessel should be marked with a wax pencil to indicate a 400 c.c. level.

Nicloux Reaction.—

Collect 400 c.c. of distillate.

Have on hand three test tubes 25 by 200 mm., with two-holed rubber stoppers, containing outlet tubes.

Tube 1.—Place 8 c.c. of brain distillate and 2 c.c. of Scotts-Wilson reagent in tube 1.

Tube 2.—This tube remains empty.

Tube 3.—Place 9 c.c. of Anstie's reagent in tube 3.

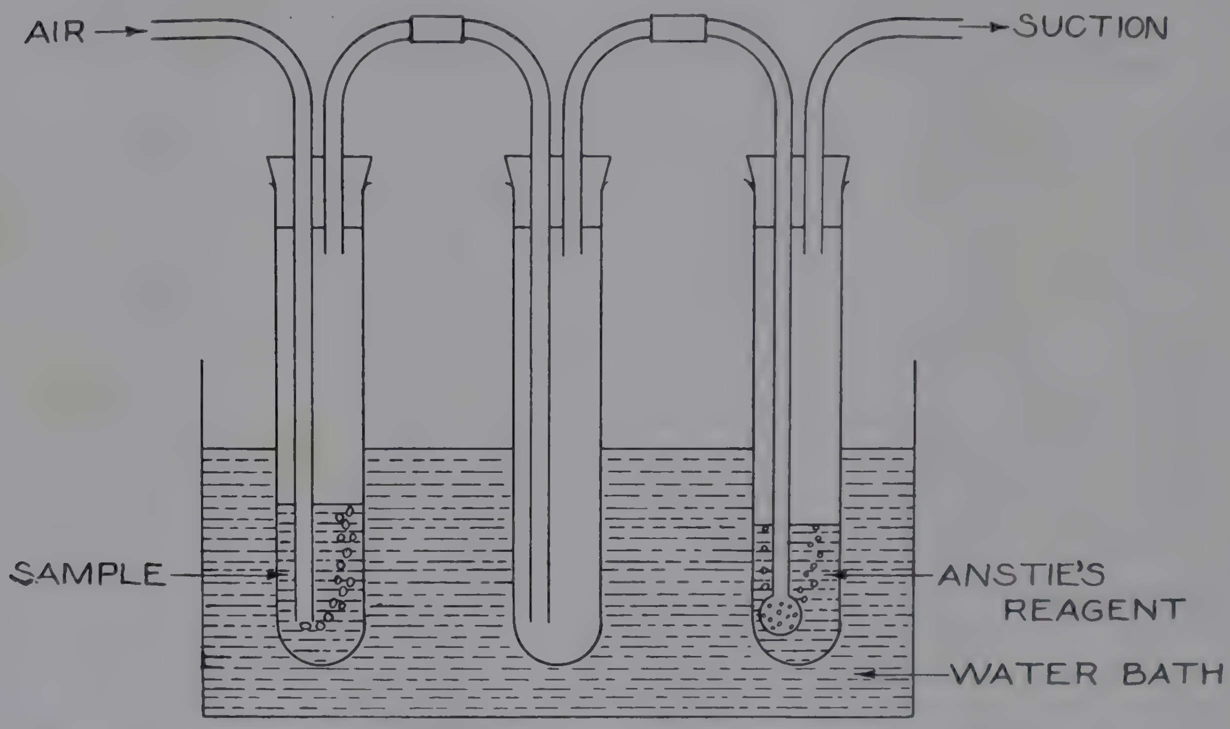

Fig. 683.—Apparatus for the Nicloux reaction. Arrange three 25 × 200 mm. tubes with two-holed rubber stoppers, and inlet and outlet tubes. The inlet tubes should extend nearly to the bottom of the tubes and the outlet tubes just below the stoppers. Connect the inlets and outlets of the three test tubes with rubber tubing in such fashion that a current of air may be aspirated through the specimen tube, then through the safety tube, into the tube of Anstie's reagent.

Stopper the three tubes.

The inlet tubing should extend nearly to the bottom of the test tubes. The outlets must be just below the stoppers.

Use rubber tubing to connect the inlet and outlet tubes.

Arrange the tubes in proper order so that a current of air may be aspirated through the specimen tube into the tube of Anstie's reagent. The second tube is to prevent foaming into the reagent. The reagent tube is placed next to the source of suction. (See Fig. 683.)

Adjust the suction so that an air current is slowly drawn from the specimen tube through the safety tube, and into the tube containing Anstie's reagent.

Immerse the tubes in a gently boiling water bath. Continue the boiling and aspiration for approximately twenty minutes.

Remove the tubes from the bath.

Cool the tube containing Anstie's reagent. Transfer the solution to a tube, similar in size and shape to the standard tubes.

Dilute to exactly 10 c.c. by adding Anstie's reagent dropwise.

Compare the color of the unknown with the color of the standards.

Report the results in milligrams of alcohol per 100 grams of tissue according to the standard tube with which the unknown matches.

Alcohol concentrations higher than 0.35 mg. per cent cannot be accurately measured with these standards since the Beer-Lampert law is not valid beyond 0.35 mg. per cent. If a concentration higher than 0.35 mg. per cent is indicated, repeat the analysis using 4 c.c. of distillate (one-half the usual quantity), and multiply the final results by 2.

Experimental.—

Various ratios of brain weight-distillate volume were tested experimentally. These experiments proved that a 1:2 ratio resulted in maximum recovery of alcohol. (Two hundred grams of brain were used to produce 400 c.c. of distillate.) Known amounts of alcohol were mixed with the alcohol-free ground brain, and analyzed for percentage recovery. Results are given in Table 219.

The results in Table 219 indicate that 100 per cent recovery of alcohol was obtained. Actually results were recorded only to the closest 0.05 mg. per cent recovery. In a few cases, the color was slightly less than the results recorded. Comparison with spectrophotometer reading revealed that even these never varied by more than 0.01 mg. to 0.02 mg. from the nearest standard report.

TABLE 219

CONCENTRATION OF ALCOHOL ADDED TO 200 GM. BRAIN	C.C. OF 2% ALCOHOL	NUMBER OF SAMPLES	ALCOHOL RECOVERED
0	0 c.c.	5	0
0.05 mg. %	5 c.c.	5	0.05 mg. %
0.10 mg. %	10 c.c.	5	0.10 mg. %
0.15 mg. %	15 c.c.	5	0.15 mg. %
0.20 mg. %	20 c.c.	5	0.20 mg. %
0.25 mg. %	25 c.c.	5	0.25 mg. %
0.30 mg. %	30 c.c.	5	0.30 mg. %
0.35 mg. %	35 c.c.	5	0.35 mg. %

Interpretation.—

Levels of 0.10 to 0.15 per cent of alcohol usually indicate that an individual is under the influence of alcohol, but not definitely intoxicated.

Levels of 0.20 to 0.25 per cent of alcohol indicate that an individual may be regarded as definitely intoxicated.

Levels of 0.30 to 0.35 per cent of alcohol indicate that the individual is quite seriously intoxicated.

Levels of 0.40 per cent of alcohol indicate that the individual is either at or quite close to the ''dead drunk'' stage. Higher concentrations indicate very serious intoxication, which may even result in death.

Determination of Alcohol in Tissues

Method of Gettler and Tiber

As soon as the tissue is removed from the body, place it in a tightly closed jar, and store in a refrigerator.

When ice cold, weigh 500 gm., quickly grind it, and place in a 2-liter flask.

Add 600 c.c. of distilled water
5 c.c. of a saturated solution of tartaric acid
and 1 c.c. of white mineral oil.

Distill with steam (Fig. 682). Use a long, well-cooled condenser.

Continue distillation until exactly 800 c.c. have been collected.

Mix the distillate thoroughly.

Place 20 gm. of potassium bichromate
and 40 c.c. of concentrated sulphuric acid
in a 500 c.c. distilling flask.

Add 300 c.c. of the distillate obtained above. Mix well.

Connect the flask to a long, well-cooled condenser by means of a Hopkins distilling head, and start the distillation.

Regulate the heat so that it will take from 45 to 50 minutes to collect exactly 250 c.c. of distillate.

Mix thoroughly.

Titrate 50 c.c. of the distillate with N/20 sodium hydroxide solution, using phenolphthalein as an indicator.

The amount of alcohol present in 1 kg. of brain tissue = (c.c. N/20 NaOH − 1.32) × 71.58. The figure 1.32 holds only for brain tissue. For other tissues, one must determine experimentally the corresponding blank.

Test to Determine Alcohol Intoxication

Considerable literature has been published regarding tests for alcohol to determine intoxication in individuals suspected of driving automobiles while intoxicated. The problem is to prove intoxication of the operator of an automobile when a driver is accused of operating while intoxicated, or for manslaughter in case death results to the victim of an automobile accident, or in a civil action for damages in which the alleged drunkenness of a defendant driver is one of the material points in issue. There are a number of tests recommended to prove intoxication, some of which are detailed in the following pages. It is evident that a knowledge of the concentration of alcohol in the blood is the best guide to prove alcoholic intoxication. Widmark, in Sweden, advocated the use of this test for blood alcohol content. He concluded that when the blood alcohol level is 200 mg. per cent or over, individuals are almost without exception intoxicated; with a value of over 160 mg. per cent and less than 200 mg. per cent, the probability of intoxication is very great. Ladd and Gibson[1] state that, on the basis of a number of European investigations, a blood alcohol level of 200 mg. per 100 gm. of blood has been recognized in Sweden and Germany as a state of intoxication. The Committee on Tests for Intoxication of the National Safety Council, 1938, recommended that blood alcohol concentrations over 0.15 per cent by weight should be conclusive evidence that a driver is under the influence of alcohol. This level is reached ordinarily by the ingestion of 6-7 ounces of whiskey. When the level is between 70 and 150 mg., the symptoms observed in the medical and physical examination should be considered. The American Medical Association's Committee on Problems of Motor Vehicle Accidents[2] made the following statement with reference to chemical tests: "Since it is impossible to diagnose drunkenness adequately—drunkenness from symptoms alone—it is important that the chemical observation of the blood, urine, saliva, and breath for alcohol be used to confirm obvious intoxication. The Committee on the Driver, of the National Safety Council, has agreed that 0.15 per cent (150 mg. per cent) of alcohol by weight in the body fluid is associated with mental and/or physical inferiority and that this figure is therefore valuable in legal cases. It must be understood, however, that much lower levels of alcohol are associated with definite impairment of judgment and particularly of self-criticism."

According to a number of European workers, there is a close agreement in the alcohol content of blood and urine after alcohol administration. Later data have shown that the urine alcohol is usually in higher concentration than blood alcohol, a ratio of about 1:1.35 having been established.

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[1] Iowa Law Rev. **24**: No. 2, Jan., 1939.

[2] Report of Committee on Problems of Motor Vehicle Accidents of the American Medical Association, J. A. M. A. **108**: 2137, 1937.

The breath alcohol test, which was recommended by Harger and which will be described later, was first brought out by Bogen.[3] His method was much improved upon by Harger.

There is a relationship of close degree between the salivary and blood alcohol levels. So far as cerebrospinal fluid is concerned, Gettler and Tiber[4] conclude that the alcohol content of the brains of post-mortem cases can be used to prove intoxication previous to death. Gettler and Freireich,[5] whose technic is described below, claim that the alcohol content of the cerebrospinal fluid, but not of the blood, can be used for estimating the amount of alcohol in the brain of a living individual. Harger, Hulpieu, and Lamb[6] denied the claims of Gettler and Freireich and believed from their experiments from dogs that brain alcohol may at all times be predicated with reasonable accuracy from the alcohol content of the blood.

There are a number of conditions affecting the predictability from these tests; for instance, tolerance and concentration of the alcoholic level. Experiments on both fasting men and animals have shown not a lessened absorption but a more rapid absorption, reaching a slightly higher peak in habituated subjects than in nonhabituated as determined in blood alcohol curves. If alcohol is administered intravenously, the curves are almost identical for both the accustomed and the unaccustomed subjects. As Ladd and Gibson (l.c.) further point out, the effect of shock, applied or mental, or of apprehension during a waiting period or during an examination may for a short time produce a sobering effect on the intoxicated individual.

Muehlberger,[7] in reference to the variations to be expected in the relationship between blood alcohol concentrations and the physical and mental impairment of different individuals, prepared a table for the National Safety Council showing the overlapping stages of intoxication. The values were as follows:[8]

1. *Subclinical:* blood 0-120 mg. per 100 c.c., urine 0-160 mg. per 100 c.c. Normal by ordinary observation; slight change by special tests.
2. *Stimulation:* blood 90-210 mg. per 100 c.c., urine 130-290 mg. per 100 c.c. Increased inhibitions; emotional instability; slight incoordination; slowing of stimuli responses.
3. *Confusion:* blood 180-300 mg. per 100 c.c., urine 260-420 mg. per 100 c.c. Disturbance of sensation; decreased pain sense; staggering gait; slurred speech.
4. *Stupor:* blood 270-390 mg. per 100 c.c., urine 380-540 mg. per 100 c.c. Marked stimuli decrease; approaching paralysis.
5. *Coma:* blood 360-480 mg. per 100 c.c., urine 510-670 mg. per 100 c.c. Complete unconsciousness; depressed reflexes; subnormal temperature; anesthesia; impairment of circulation; stertorous breathing; possible death.

Precautions and Methods in Respect to the Blood Test.—Mason and Ladd emphasize the legal side of the method of taking the blood test. They state that care should be taken that the physician-patient relationship is not established so as to bring the test within the scope of privileged communications and observations. The doctor taking the blood specimen should avoid rendering any medical treatment at the time, as that would give rise to the privilege in some states. If necessary to give first aid treatment, some physician other than the one taking the blood specimen should give this treatment. The physician taking the test should also note the odor of alcohol on the breath, abnormalities of the gait and speech, size of the pupils, flushing of the face, and should be able to state that the patient, in his opinion, is not perceptibly, or is slightly or highly affected by alcohol. The specimen should be taken after proper disinfection of the skin. The use of alcohol, alcohol preparations, or phenol solution for sterilization of the skin might affect legally the validity of the analytical results. To avoid possibility of civil action, subject's consent should be obtained. Blood should be withdrawn in the usual manner for blood chemical test into a bottle containing sodium fluoride. Stopper the container with a clean cork and mix. Samples should be sealed and labeled in the presence of a witness, showing the name of the donor, hour and date the specimen was taken, and the signature of the physician and witness or witnesses. Specimen should be delivered personally by the physician to the chemist or qualified person who is to make the analysis. If specimens are mailed to a laboratory, they should be registered or sent by special delivery so that receipt by the analyst is verified by signature. Specimen should be identified and marked. Both blood and urine specimens should be taken.

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[3] Drunkenness: A Quantitative Study of Acute Alcoholic Intoxication, J. A. M. A. **89**: 1708, 1927; Am. J. M. Sc. **176**: 153, 1928.

[4] Arch. Path. Lab. Med. **3**: 218, 1927.

[5] J. Biol. Chem. **92**: 199, 1931.

[6] J. Biol. Chem. **120**: 689, 1937.

[7] Cited from Committee on Tests for Intoxication of the National Safety Council.

[8] Ladd, Mason, and Gibson, R. B.: L. c.

Special precautions with respect to the glassware used in analysis should be carried out; that is, it should be chemically cleaned and thoroughly rinsed with pure distilled water.

Just what test is to be used depends on the individual and the experience of the analyst. The method of Gettler and Freireich and the chemical test of breath by Harger are detailed in the following pages.

Determination of Ethyl Alcohol in Blood and Spinal Fluid

(Method of A. O. Gettler and A. W. Freireich*)

Ten c.c. of spinal fluid and 1 gm. of paraffin are placed in a 100 c.c. distillation flask which is set up for steam distillation (Fig. 682). It is to be noted that the flask containing the blood or spinal fluid is not heated, but steam alone is used for the distillation. One hundred c.c. of distillate are collected. To these 100 c.c. of distillate, placed in a 200 c.c. round-bottom flask, are added 7.5 gm. of potassium dichromate and 15 c.c. of concentrated sulphuric acid. Connect the flask to a long, well-cooled condenser by means of a Hopkins distilling head. The contents are well mixed.

A medium-sized (1½ inch) Bunsen flame is applied in order that the distillation is not too rapid. It should be slow but at a constant rate and should take about 60 minutes to collect the required 80 c.c. of distillate.

After collecting 80 c.c. of distillate, it is mixed and aliquot portions are titrated with 0.05 N sodium hydroxide for acetic acid produced.

The titration is carried out by using phenolphthalein indicator. The indicator is added to the distillate, the 0.05 N sodium hydroxide is placed in a buret, and the alkali added to the distillate a drop at a time with constant stirring until the first faint permanent pink color appears. This is the end point.

The amount of sodium hydroxide (0.05 N) used is read from the buret. This is the equivalent of the 0.05 N acetic acid in the distillate.

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*J. Biol. Chem. 92: 199, 1931.

The amount of alcohol in the spinal fluid is calculated according to the following figures:

$$\text{Per cent of alcohol} = (\text{c.c. } 0.05 \text{ N alkali used} - 0.42) \times \frac{100}{85} \times 0.0023 \times 10.$$

The figure 0.42 represents the blank control to eliminate the normal amount of titratable acid in spinal fluid.

$\frac{100}{85}$ is the factor used based on the fact that, from normal spinal fluids to which alcohol was added, only 85% of the added alcohol was recoverable by this method.

0.0023 represents the grams of alcohol equivalent to 1 c.c. of 0.05 N alkali used in the titration.

10 is the multiple used to convert to 100 c.c. of spinal fluid, because 10 c.c. of spinal fluid were used in the titration.

A small portion of this distillate is now tested for the sulphate ion to make sure that no sulphur trioxide passed into the distillate.

From the amount of acetic acid found by titration, the amount of ethyl alcohol is calculated (above).

It was found by Gettler and Freireich that nonalcoholic spinal fluid yields certain volatile substances to the distillate, which, when oxidized in accordance with their method, yield on the average for every 10 c.c. of spinal fluid an acidity of 0.78 c.c. 0.02 N, which is equivalent to 0.0071 per cent of ethyl alcohol, or 0.71 mg. of ethyl alcohol in every 10 c.c. of spinal fluid.

In order to determine whether normal blood yields any acidity, the method was carrried out by these investigators on fourteen patients who had been under observation in the hospital and had partaken of no alcohol. These experiments showed that nonalcoholic bloods yield certain volatile substances to the distillate, which, when oxidized by their method, yield for every 10 c.c. of blood an acidity of 1.33 c.c. or 0.012 per cent of ethyl alcohol. They do not claim that this is ethyl alcohol, but that it is some volatile material found in normal blood which, upon oxidation, yields titratable acidity.

These workers further examined the alcohol content of the brain in a series of cases and showed that the alcohol content of the brain can be used to prove intoxication. In order to draw conclusions, they examined the brain, blood, and spinal fluid of cadavers, and the blood and spinal fluid of living alcohol drinkers, and determined the relationship between the alcohol of the brain and that of the spinal fluid. They showed that the smaller the alcohol content of the brain, the larger is the spinal fluid:brain ratio. The larger the amount of alcohol in the brain, the nearer do the spinal fluid values approach the values in the brain, the spinal fluid: brain ratio approaching a value of 1.1. On the other hand, the spinal fluid: brain ratio in intoxicated individuals is below 1.4. They showed also that the blood: brain ratios range anywhere from 0.66 to 4.55.

From their figures, they deduced that the *spinal fluid, but not the blood,* can better be used for estimating the amount of alcohol in the brain of a living individual. The alcohol content of the brain and that of the spinal fluid run quite parallel; since the alcoholic content of the brain is an index of intoxication, *the spinal fluid can be used to prove intoxication in living individuals.*

They give the figure of 0.272 per cent or more alcohol in the spinal fluid to show that an individual is intoxicated. All figures below 0.2 per cent were non-intoxicated individuals.

Chemical Tests of Breath for Alcoholic Intoxication

The Harger Drunkometer

A rapid chemical test for intoxication employing the breath was described by Harger, Lamb, and Hulpieu.[1] The method utilizes apparatus called the Harger Drunkometer. The test gives prompt information; one can determine within a few minutes whether a person has been drinking, and if so, the approximate accumulation of alcohol in his body. The preliminary test is easily performed and does not require specialized chemical knowledge. The final test, however, which is quantitative, requires the services of a chemical expert. The test has been accepted in many states. The test for alcohol depends on a change in color, with the disappearance of the initial purple, produced by the combination of 16 N sulphuric acid and N/20 potassium permanganate solution, mixed in a glass portion of the Drunkometer into which is allowed to flow air from a balloon which has been blown up by the subject. The details of the apparatus insofar as the quantitative analysis is concerned are seen in Fig. 684.

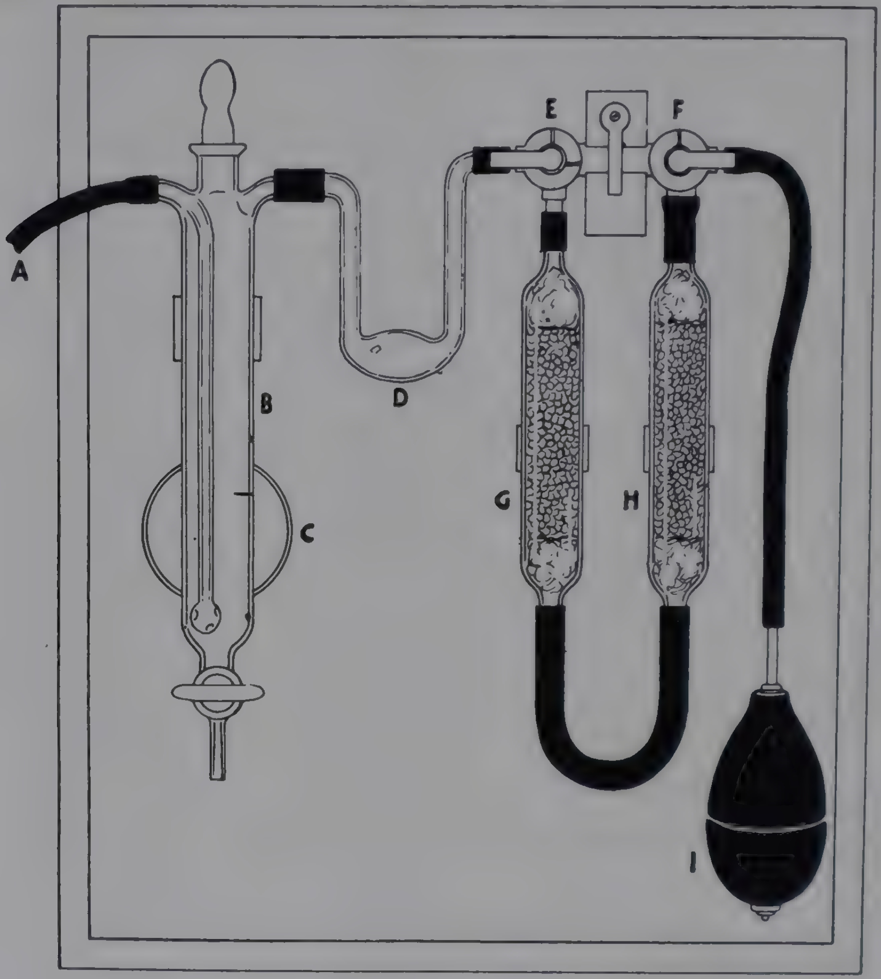

Fig. 684.—Details of the Harger Drunkometer. The reagent used is a weak solution of potassium permanganate in the presence of approximately 55 per cent sulphuric acid by weight.

The technic is as follows: (Fig. 684.) Turn valves *E* and *F* to shunt the gas past tube *G* and directly out by way of the rubber tube and bulb I. In the reaction tube *B* place 10 c.c. of solution I, then exactly 1 c.c. of solution II,* solution I being 16 N sulphuric acid and solution II, N/20 potassium permanganate solution; replace stopper and mix contents by one or two strokes of the bulb pump.

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*This mixture deteriorates rapidly and loses its purple color; it should be used within 5 or 10 minutes of the time it is prepared.

[1] J. A. M. A. **110**: 779, 1938.

With the subject's cooperation, collect slightly more than 2 liters of breath in one of the balloons. Connect the balloon to a glass adapter the other end of which is connected to a rubber tube containing a screw clamp. Close the clamp. Release air from balloon until it fits the large 2 liter ring. Immediately connect the rubber tube of the adapter to inlet tube *A* (Fig. 684) by means of a small glass nipple, open the pinch clamp, and allow the breath to pass fairly rapidly through the apparatus. If the solution in Tube *B* is not decolorized by 2 liters of breath, the subject's blood contains less than 0.35 mg. of alcohol per c.c., if any, and he may be discharged as not having been drinking, at least to an appreciable extent.

If less than 2 liters of breath decolorizes the purple liquid, measure the quantity of breath used in the test by comparing the diameter of the balloon containing the remaining breath with the two smaller brass rings. If more than 1,000 c.c. were used, subject's blood contains less than 0.7 mg. of alcohol per c.c. and he is probably not under the influence of alcohol. If less than 1,000 c.c. of breath decolorizes the liquid, perform the quantitative test (below). The end point is the disappearance of the last trace of purple leaving a faint yellowish brown fluid.

Having determined the positive presence of alcohol in the breath by decolorization of the reagent mixture, a quantitative determination of the amount can be made by permitting the air to flow into the tube containing ascarite for the absorption of carbon dioxide. The three-way metal valves (*E* and *F*, Fig. 684) are so arranged as to leave tubes *G* and *H* out of the circuit or to send the gas stream successively through them.

Quantitative Test.—Weigh tube *H* and connect in position, the valves *E* and *F* being turned so as to include tubes *G* and *H* in the path of the gas. Use either the balloon or the pump to collect the breath, and proceed as above. When the last trace of purple has just disappeared from the fluid in tube *B*, replace tube *H* at once in its container and close the container. Send tube *H* to the laboratory for weighing. If the balloon is used, measure accurately the volume of breath passed through the apparatus by disconnecting the rubber bulb and connecting the outlet tube to a gas buret filled with water.

Calculations.—Under the above conditions, 1 c.c. of N/20 potassium permanganate is just decolorized by 0.175 mg. of ethyl alcohol. The weight of carbon dioxide absorbed by tube *H* gives the amount of alveolar air which contained 0.175 mg. alcohol. Calculate the quantity of alcohol which accompanied 190 mg. of carbon dioxide; that is, the weight of alcohol in 2 liters of alveolar air, and therefore the weight of alcohol in 1 c.c. of the subject's blood. Let x equal milligrams of alcohol in 2 liters of alveolar air and w the increase in weight of tube *H* in milligrams.

$$\frac{x}{190} = \frac{0.175}{w} \quad \text{or} \quad x = \frac{33.25}{w}$$

To calculate the milligrams of alcohol in 2 liters of alveolar air in 1 c.c. of the subject's blood, divide 33.25 by the weight of carbon dioxide contained in the sample of breath which just decolorized the purple fluid.

To check this figure, calculate the weight of alcohol in 4 liters of the sample of breath (observation of Borgen that the breath as collected usually contains about 50 per cent of alveolar air). It will require 560 c.c. of breath containing 1 mg. of alcohol in 4 liters to decolorize 1 c.c. of permanganate solution. Then 560 ÷ c.c. breath used = mg. of alcohol in 4 liters of expired air or in 1 c.c. of blood.

The ratio of alcohol to carbon dioxide in the breath may be used to measure the concentration of alcohol in the blood. The weight of the alcohol accompanying 190 mg. of carbon dioxide in the breath is very nearly equal to the weight of the alcohol in 1 c.c. of the subject's blood. Employment of the ratio of alcohol to carbon dioxide in the breath permits the test to be done without the subject's being touched. A tube is held in the breath stream and a pump draws the sample through the apparatus. As shown by Harger's test, 4 liters of expired air contained about the same amount of alcohol as 1 c.c. of the subject's blood. Because of possible fluctuations in the amount of alveolar air in such samples, it is believed that analyses of breath made on the volume basis should be checked by determining the carbon dioxide in the sample.

Fig. 685 covers 120 tests made on individuals in the City Hospital of Indianapolis, showing the correlation of breath and blood tests. This indicates very clearly that the breath test is a safe and accurate method for estimating whether or not a given motorist is capable of safely driving a car.

Certain precautions are to be borne in mind in the quantitative test. First, ascarite tube (*H*) must be kept free from moisture when it is not in use. It should be stored in a larger glass tube containing a mixture of ascarite and dehydrite and closed with a paraffin cork. If tube *H* is not in position, close the open end of the rubber tube at the bottom of tube *G* by means of a glass or metal plug to prevent entrance of moisture into tube *G*.

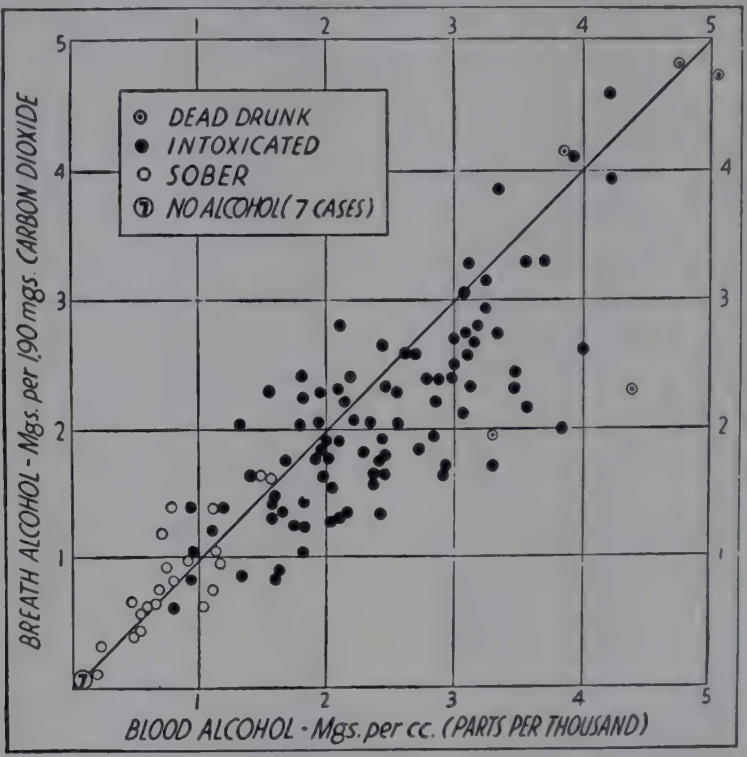

Fig. 685.—Correlation of breath alcohol and blood alcohol in tests in 120 individuals.

It is also a good idea in connection with the use of the Drunkometer to have what is known as an "artificial drunk." This is to familiarize the operator with the end point of the reaction and to demonstrate the reaction in court. For this purpose have a bottle fitted with a two-hole rubber stopper, one hole carrying a glass inlet tube extending to the bottom of the bottle, and the second a glass ell which ends just below the stopper. To obtain an "artificial drunk," place a weak (0.5%) solution of alcohol in the bottle and draw air through the solution. Use a second bottle fitted like the first for making qualitative tests for alcohol in the urine, since the urine of alcoholic subjects will serve as the fluid in the "artificial drunk."

The Intoximeter

This is another method of determination of the presence and amount of alcohol in the breath by means of an apparatus originated by Glenn C. Forrester.[1] This method has the advantage of being more easily portable and yields approximately as good results as the Harger Drunkometer.

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[1] For further details, see book by Glenn C. Forrester, issued by Intoximeter Association, 334 Buffalo Avenue, Niagara Falls, New York, entitled *Use of Chemical Tests for Alcohol.*

This instrument is housed in a cylindrical mailing tube, 2 inches in diameter and 8 inches long. It enables a police officer to obtain an on-the-spot approximation of the level of blood alcohol by permitting the breath, which is exhaled into a rubber balloon, to pass through a mass of sintered glass particles within a capillary tube moistened with a purple oxidizing solution, and noting the time required to destroy the color. In the presence of alcohol, the color disappears, and the number of seconds, by reference to a table on the wrapper, indicates the percentage of blood alcohol. No disappearance of color means no alcohol. If this preliminary test is positive, the police officer or technician permits some of the air to pass from the balloon, while the color test is being conducted, through two tubes in series housed in the case. Tubes are sealed securely when this air has passed. The tubes are then transported to the laboratory where a chemical test is made. The weight of CO_2 is determined by direct weighing of the ascarite tube on an analytical balance. This weighing is made by the same chemist who weighed the tube before use, recorded that weight, and sealed the record and tube into the Intoximeter before issuing it to the officer.

The second tube contains perchlorate. Recommended procedure with this tube is to mix it with 40 to 60 c.c. of doubly distilled water. Twenty-four c.c. are distilled into a glass stoppered graduated flask, and the volume is made up to exactly 25 c.c. This is then well mixed and exactly 10 c.c. pipetted into a clean 25 by 150 mm. Pyrex test tube, previously charged with exactly 5 c.c. of 0.0438 N. potassium dichromate solution (1.705 grams of pure dry reagent potassium dichromate dissolved in one liter of solution) and with approximately 5 c.c. of reagent quality sulphuric acid. These two have been previously mixed and cooled by placing in a beaker of cold water. Swirl test tube with 20 c.c. of contents to mix contents thoroughly. Heat in a boiling water bath for about 15 minutes to complete the oxidation of the alcohol to acetic acid.

Cool the tube and transfer contents into a clean 500 c.c. Erlenmeyer flask and dilute to 200 c.c. total volume with distilled water. Add 2 grams of reagent quality potassium iodide and titrate the liberated iodine with sodium thiosulphate solution (0.00434 N, made by dissolving 1.1 grams of thiosulphate crystals in 1 liter of water and preserving with a few drops of chloroform).

Determine end point with 1 c.c. of starch indicator solution (preserved with a crystal of mercuric chloride).

A blank determination is run on the magnesium perchlorate of the same batch used in the preparation of the Intoximeter and distilled in the same way as in the regular analysis. This blank amounts to about 0.2 c.c. of sodium thiosulphate solution.

Colorimetric Method for Determination of Alcohol Concentration in Urine and Blood

Method No. 1

A simple method for determination of alcohol concentration in urine and blood has been described by Sheftel,[1] as follows:

Apparatus and Reagent Required.—

50 c.c. Pyrex Erlenmeyer flask.

One rubber cork in which a Monel or nichrome wire clip is inserted to keep the filter paper in place.

Filter paper strips of medium thickness, 1½ by 2 inches.

Special blue filter.*

Accurate 0.2 c.c. pipette.

Reagent.—

Solution of 0.4262 per cent of potassium dichromate in 50 per cent (by volume) sulphuric acid (1 c.c. of this reagent corresponds to 1 mg. of alcohol).

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*Lantern blue glass filter No. 554, manufactured by MacGregor Instrument Co., of Needham, Mass.

[1] J. Lab. & Clin. Med. 23: 534, 1938.

Procedure.—

Roll one filter paper strip into a small cylinder and insert it into the clip attached to the cork. Introduce into the Erlenmeyer flask 1 c.c. of reagent. Absorb into the filter paper 0.2 c.c. of urine or blood to be examined.

The blood may be oxalated or freshly drawn from the ear lobe or the tip of the finger. Insert the cork immediately, but not tightly into the Erlenmeyer flask. Heat the neck of the flask from all sides over a Bunsen burner, and then press the cork very tightly into the neck of the flask. Place in a one liter beaker a small quantity of water, and into this put the flask. Adjust the volume of water in the beaker so that the flask does not float. Cover the beaker with a Petri dish or a watch glass. Boil the water in the beaker gently for fifteen minutes. Remove the flask from the beaker. Remove the cork gently from the flask, cool lower portion under tap, and add 4.3 c.c. of distilled water. Into a clean test tube introduce 2.5 c.c. of reagent and add to it 10 c.c. of distilled water. It will be seen from this that whereas 1 c.c. of reagent standard is diluted to 5 c.c., 1 c.c. of unknown is diluted to 5.4 c.c. Approximately 0.1 c.c. of the blood remains on the filter paper as unevaporated material.[1] The difference in the dilution is the necessary correction for absorption of light in the colorimeter by chromic sulphate. Compare in any microcolorimeter, using the blue filter and setting the unknown at 10. Calculate from the following formula:

$$\left(1 - \frac{R}{10}\right) \times 500 = \text{mg. alcohol per 100 c.c.}$$

R = Reading of the reagent-standard.

For example: R is 7.4; then $\left(1 - \frac{7.4}{10}\right) \times 500 = 130$ mg. alcohol per 100 c.c.

When the concentration of alcohol in blood or urine is high (above 250 mg. per cent), it is advisable to dilute the reagent standards by 2 or 4.

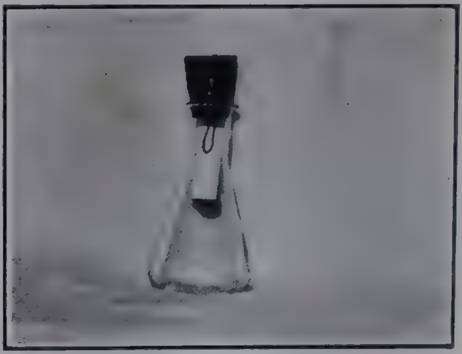

Fig. 686.—Sheftel apparatus for determination of alcohol concentration in urine and blood. (From Sheftel, A. G.: J. Lab. & Clin. Med., Feb., 1938.)

Method No. 2

The following method is in use in a number of crime laboratories.

Reagents.—

Picric Acid Solution, half-saturated.—

Dilute saturated picric acid to double its volume with distilled water.

Modified Anstie's Reagent.—

Dissolve	3.3333	gm.	of potassium dichromate, c.p.
in	250.0	c.c.	distilled water
Add	521.0	c.c.	concentrated sulphuric acid
to	200.0	c.c.	distilled water in a Pyrex flask, cooling under running water.

When cool, add the dichromate solution, slowly and with constant shaking. Dilute to 1000 c.c. with distilled water.

Apparatus.—

250 c.c. Erlenmeyer flask with glass tubing bent to connect with condenser, or 250 c.c. distilling flask. Condenser: either Liebig or Graham coiled (latter preferred).

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[1] Abels, J. C.: Proc. Soc. Exper. Biol. & Med. 34: 346, 1936.

Rubber tubing for condenser connections.
Rubber stoppers with one hole for glass tubing connections.
Bunsen burner.
Centrifuge tube, graduated.
15 c.c. test tubes, ⅝ inch diameter.
1 set of color standards.
1 c.c. pipette.
10 c.c. pipette.

Technic.—

Pipette 10 c.c. of blood or urine into a clean, dry 250 c.c. Erlenmeyer flask.

Add 10 c.c. half-saturated picric acid solution.

Connect the flask to the condenser. **CAUTION:** Be sure that all stoppers fit tightly so that no steam can escape anywhere.

Apply heat gently. When using blood, glass beads should be used to prevent bumping.

Collect 10 c.c. of distillate in the 15 c.c. centrifuge tube. **NOTE:** If less than 10 c.c. of blood or urine is used, then the aliquot portion is collected.

To 3 c.c. of Anstie's reagent, carefully measured with a test tube*
add 1 c.c. of distillate delivered from a clean dry 1 c.c. pipette.

Place the tube in boiling water bath for 4 minutes.

Compare the color against standards and read directly. NOTE: A set of standards may be purchased from the LaMotte Chemical Company, Baltimore, Md., or may be made in the laboratory.

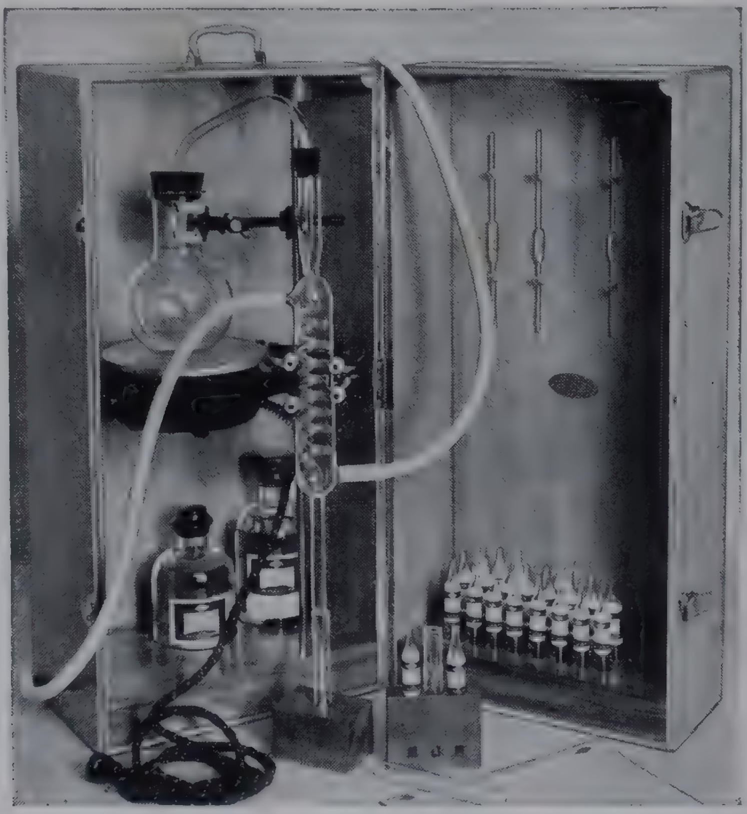

Fig. 687.—The LaMotte apparatus for determining concentration of alcohol in blood and urine. (Courtesy LaMotte Chemical Products Company, Baltimore, Md.)

LaMotte Outfit for Determining the Concentration of Alcohol in Blood and Urine[1]

This is a simple and specific test for alcohol in body fluids.

In operating the test the alcohol is distilled from a special acid mixture. The color which is developed by reaction of the distillate with standard potassium dichromate solution is compared with permanent color standards to obtain the percentage of alcohol in the fluid under examination.

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*If the LaMotte Standards are used, the ⅝ inch test tube should be used since it is of the same bore.

[1] Heise, H. A.: Am. J. Clin. Path. 4: 182, 1934.

DETERMINATION OF METHYL ALCOHOL IN TISSUES

Gettler[1] made the subject of an investigation fifty-eight tests for methyl alcohol found in the literature. It was found by him after an analysis of over 250 liquors and over 700 human organs that some of these tests are unreliable and that foreign substances present have marked influence upon them. He therefore worked out a typical procedure which is quite reliable. His method follows:

Finely grind 500 gm. tissue, and place in a 1.5 liter distilling flask.

Add 500 c.c. water and enough sulphuric acid to make it distinctly acid.

Add a few drops of mineral oil to prevent frothing.

Distill with steam (Fig. 682). The distilling flask containing the material must be heated on a boiling water bath during steam distillation.

Collect 300 c.c.

Place the distillate in a 500 c.c. distilling flask, neutralize to phenolphthalein, if necessary, and slowly redistill, this time without steam.

Collect 100 c.c. and put into a 150 c.c. distilling flask.

Add 5 c.c. concentrated sulphuric acid and allow to cool.

Add 0.1 gm. potassium dichromate and shake to dissolve. Connect with the condenser and receiver and slowly distill until 40 c.c. are obtained. All the formaldehyde usually comes over in the first 40 c.c. unlike that in the distillation of the oxidized liquors. Use 2 c.c. of the distillate for each of the first five tests, and 30 c.c. for the sixth.

1. Fuchsin (reduced) plus sulphuric acid gradually develop a violet red color. If no sulphuric acid is added, both acetaldehyde and formaldehyde give the reaction.

2. Phenylhydrazine hydrochloride plus sodium nitroprusside plus sodium hydroxide yield a deep blue color.

3. To 3 c.c. of distillate add 5 c.c. of concentrated sulphuric acid; mix; cool. Then add a few mg. of morphine. A violet red color develops if methyl alcohol was originally present in this case.

4. To 3 c.c. of distillate add 3 c.c. of milk, then add a drop of very dilute ferric chloride and 5 c.c. of concentrated hydrochloric acid. Place the mixture in a boiling water bath. A violet color develops if methyl alcohol was originally present in the tissue examined. Although this test is unreliable when examining liquors, it is quite trustworthy and characteristic when working with tissues. This is probably due to the organism having destroyed or conjugated and excreted the interfering substance originally present in liquors.

5. To 3 c.c. of distillate add 5 or 6 drops of a saturated alcoholic solution of gallic acid and stratify some concentrated sulphuric acid underneath the mixture. A green to blue ring develops if methyl alcohol was originally present in the tissue.

6. Concentrated ammonia condenses with formaldehyde on evaporation into hexamethylenetetramine (urotropine). The latter gives characteristic crystals with mercuric chloride.

It is not necessary to obtain hexamethylenetetramine-mercuric chloride crystals to prove conclusively the presence of methyl alcohol in tissues. The reason for this is that the foreign substances found in liquors which cause false reactions are oxidized or conjugated and excreted rapidly from the organism, so that, on distillation of the brain or liver tissue, they are absent in the distillate.

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[1] J. Biol. Chem. **43**: No. 2, June, 1920.

Rapid Method for Determination of Methyl Alcohol in the Blood and Body Fluids (Ozburn Method)

Ozburn[1] described a new test for methyl alcohol that is sensitive, specific, using stable and readily obtained reagents. It will detect as little as one microgram in one milliliter of water and easily has detected as little as two-tenths microgram of methyl alcohol in 1 c.c. of blood. Most procedures for the detection of methyl alcohol depend on its oxidation to formaldehyde and this method is no exception. The formaldehyde is usually determined by means of Schiff's reagent which is a solution of rosaniline decolorized by standard sulphurous acid in some form.* This reagent is unstable and pure quality rosaniline is difficult to obtain. Chromotropic acid and sodium bisulphite, both solids, are stable and easily procured in pure form. The advantages of these reagents over Schiff's is easy to understand. The concentrated sulphuric acid provides sufficient heat for the color reaction and vitiates the need for a water bath. The test is more specific than that with Schiff's reagent and the color produced is more stable, persisting 48 hours or more. The following substances have been tested for their possible interference in this test for methyl alcohol: ethyl alcohol, acetone, sodium formate, aspirin, Phenacetin, acetanilid, sodium barbital, sodium Amytal, phenobarbital, Nembutal, Seconal, procaine hydrochloride, cocaine hydrochloride, caffeine, atropine, quinine, Atabrine, nicotine, chloral hydrate, salicylic acid, sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine. None give a positive test immediately, although nicotine, codeine, and quinine give a positive reaction upon standing overnight. Old solutions of cocaine, however, give a positive test, while fresh solutions do not. Methenamine (urotropin) gives an intense positive reaction, as would be expected.

Reagents.—

Sodium Bisulphite, $NaHSO_3$, reagent grade.

Chromotropic Acid, 1,8-dihydroxynaphthalene 3,6-disulphonic acid (Eastman Kodak Co., No. 1613).

Potassium Permanganate Mixture.—

Dissolve 15 gm. potassium permanganate
 and 75 c.c. of 85% phosphoric acid (H_3PO_4)
in distilled water and dilute to 500 c.c. with distilled water.

20% Trichloroacetic Acid.—

Dissolve 20 gm. of trichloroacetic acid in distilled water and dilute to 100 c.c. with distilled water.

Concentrated Sulphuric Acid, reagent grade.—

Technic.—

Place 2 c.c. of oxalated blood in a 50 c.c. Erlenmeyer flask.

Add 2 c.c. of 20 per cent trichloroacetic acid slowly with constant shaking.

Stopper the flask and shake well for 1 minute.

Pour contents into a 15 c.c. centrifuge tube and whirl for 5 minutes at 2000 r.p.m.

If the supernatant liquid is clear, carefully pipette 1 c.c. into a small test tube. If it is not clear, pour through a small No. 42 Whatman filter paper and use 1 c.c. of the filtrate.

Add 0.2 c.c. of the permanganate reagent and let stand for 1 minute, shaking gently from time to time.

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*See page 2260.

[1] U. S. Naval Med. Bull. 46: 1170, 1946.

Add solid powdered sodium bisulphite, using just enough to decolorize the permanganate completely and also to decolorize any brown manganese dioxide that is formed. The amount of sodium bisulphite is usually about 10 mg., and the amount which will just cover the butt end of any ordinary pencil will usually suffice.

Add chromotropic acid powder and shake gently. The amount of chromotropic acid which can be lifted on the end of an ordinary safety match is sufficient.

Add 1.5 c.c. of sulphuric acid, tilting the tube and allowing the acid to flow down the sides of the tube so that it forms a separate layer underneath the solution. At this point, carefully examine the junction between the aqueous solution and the underlying sulphuric acid. If methyl alcohol is present, a purple ring will be seen at this junction.

Cautiously mix the contents of the tube by gently swirling it.

Let stand until cold.

If methyl alcohol is present, a diffuse violet color will be produced, the intensity of which will be proportional to the concentration of methyl alcohol.

Blood filtrates having no methyl alcohol give a light brown tint.

Those using the test should make preliminary determinations on several known positive and negative specimens to familiarize themselves with the procedure and the colors. Whenever possible, control positive and negative specimens should be run with each unknown.

Qualitative Test for Methyl Alcohol Using the Folin-Wu Blood Filtrate

Wright-Elvove Method Modified by Harger[1]

This is a modification of the well-known Wright-Elvove method. Harger found that the addition of a little ethyl alcohol to the fluid being tested greatly increases the sensitivity of the method when dealing with small quantities of methyl alcohol. This makes it possible to do the test directly on the Folin-Wu protein-free filtrate from blood (page 224), and thus avoid a distillation. The reagents are those described by Wright,[2] and are as follows:

Reagents.—

Potassium Permanganate.—

Dissolve 3 gm. potassium permanganate
in 100 c.c. distilled water (previously distilled over potassium permanganate) and containing 15 c.c. phosphoric acid.

By using water previously distilled from potassium permanganate solution, this reagent can be kept for a long time.

Oxalic Acid Solution.—

Dissolve 5 gm. of oxalic acid
in 100 c.c. of 50% sulphuric acid. (Dilute 50 c.c. of sulphuric acid, sp. gr. 1.84, to 100 c.c. with distilled water, adding the acid to the water in a Pyrex flask.)

Schiff's Reagent.—

Dissolve 0.2 gm. rosaniline (or equivalent weight of its salt)
in 120.0 c.c. hot distilled water.
Cool.

Dissolve 2 gm. of sodium bisulphite (metabisulphite) in 20 c.c. distilled water.

Add the rosaniline solution to the bisulphite solution, and add to the whole 2 c.c. concentrated hydrochloric acid.

Dilute the reagent to 200 c.c. with distilled water.

The reagent should become colorless or nearly so upon standing. No deterioration results if it is protected from the air. Use a very pure rosaniline base.

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[1] Personal communication.

[2] J. Indust. & Eng. Chem. 19: 750, 1927.

Technic.—

Place 2 c.c. of Folin's protein-free filtrate (page 224) in a test tube, and add 1 drop of 95% ethyl alcohol.

Add 1 c.c. of potassium permanganate solution. Allow to stand for ten minutes, then decolorize by adding 1 c.c. of the oxalic acid solution.

Add 2 c.c. Schiff's reagent, and mix. The solution must be thoroughly mixed.

If methanol is present, a violet color develops. With traces of methanol, the color is not developed for an hour.

For approximate quantitative results, a comparison may be made with various standard solutions of methyl alcohol in water treated in the same manner. A convenient standard contains 0.2 mg. methyl alcohol dissolved in 2 c.c. water. In cases of fatal methyl alcohol poisoning as much as from 0.5 to 1.5 mg. of methyl alcohol per c.c. of blood was found by Harger.

For smaller amounts of methanol a distillate of the protein-free filtrate may be employed, distilling 2 c.c. from 20 c.c. of the protein-free filtrate.

DETERMINATION OF ALCOHOL IN LIQUORS

Per Cent by Volume and by Weight

Assemble distilling apparatus of appropriate size.

Pack the receptacle in ice. The receptacle of choice is a volumetric flask which is to be used as the standard measure of the final volume of distillate.

Preparation of Specimen

Whiskey and Gin.—

Dilute 10 c.c. of the sample to 200 c.c. with distilled water. Distill and collect 100 c.c. of the distillate. On the basis of per cent, or 100 c.c., using 10 c.c. of the sample gives a correction factor of 10 (the alcohol distills over before the water, consequently all the alcohol from the 10 c.c. sample will be in the distillate).

Wines.—

Dilute 25 c.c. of the sample to 200 c.c. with distilled water and proceed as for whiskey and gin, above. The factor for calculation is 4.

Beer.—

Dilute 100 c.c. of the sample to 200 c.c. with distilled water and proceed as for whiskey and gin. The factor for calculation is 1.

Determination of Alcohol

Obtain the density of the distillate by using either the hydrometer or the pycnometer. Use a constant temperature, preferably 20° C.

Use of Pycnometer.—Obtain the weight of the empty pycnometer (with stopper). Completely fill the pycnometer with distilled water at 20° C., insert stopper, and obtain the weight. Subtract the weight of the empty pycnometer from the weight of the pycnometer filled with water to obtain the weight of the distilled water. Now clean and dry the pycnometer, and completely fill it with the distillate. Obtain the weight. Subtract the weight of the empty pycnometer from this figure to obtain the weight of the distillate. Figure the specific gravity of the distillate by using the following formula:

$$\frac{\text{Weight of distillate}}{\text{Weight of the distilled water}} = \text{Specific gravity of the distillate.}$$

Calculation.—

Consult standard tables for conversion of specific gravity of alcohol to per cent by volume and by weight. Such tables are found in many handbooks of physics and chemistry.*

Multiply by the correction factor as given above.

Report in both per cent by volume and per cent by weight.

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*Handbook of Chemistry and Physics, published by the Chemical Rubber Publishing Company, Cleveland, Ohio, contains these tables.

TESTING OF WHISKIES FOR IMPURITIES

Coloring

Shake together 5 c.c. of the sample and 5 c.c. of chloroform.

A clear color in the chloroform suggests artificial coloring.

The natural color will be in the chloroform.

Fusel Oil

To 10 c.c. of the sample, add 10 drops of colorless aniline and 3 drops of sulphuric acid.

A deep red color is formed if fusel oil is present.

DETECTION OF METHYL ALCOHOL IN LIQUORS[1]

Preparation.—

Place 100 c.c. of liquor in a 200 c.c. distilling flask, neutralize with sodium carbonate solution, using phenolphthalein as indicator, and slowly distill until 50 c.c. of distillate have been collected.

Oxidation.—

Add 100 c.c. of 10% sulphuric acid to distillate, using a 250 c.c. distilling flask; also add 6 gm. of potassium dichromate and let stand for 10 minutes. Connect with a condenser and receiver and very slowly distill off 30 c.c.

The distillation of these 30 c.c. should take 1 hour; the distillate then contains most of the acetaldehyde, but very little of the formaldehyde, and is to be rejected. The distillation is then continued a little faster, until 60 c.c. are obtained. This part of the distillate contains most of the formaldehyde and hardly any acetaldehyde. Some formaldehyde may still be left in the distilling flask, but the greatest portion is in these 60 c.c. and this is used for the tests.

Detection.—The tests are conducted in the following sequence:

1. To 2 c.c. of this oxidized fraction add 10 drops of a 5% solution of phenylhydrazine hydrochloride, 1 drop of 0.5% sodium nitroprusside solution, and 10 drops of 10% sodium hydroxide solution. If formaldehyde is present, a blue color is obtained which changes to green and then to yellowish red. Acetaldehyde produces a red color.

2. To 2 c.c. of the material add 2 drops of 2% phenol and carefully stratify sulphuric acid underneath. A red ring develops. Acetaldehyde gives a yellowish green, brown, or reddish brown ring.

3. To 2 c.c. of the oxidized material, add 5 c.c. of concentrated sulphuric acid, then add a few mg. of apomorphine. A reddish violet color develops.

4. Add 0.08 gm. peptone, 3 drops of 3% ferric chloride, and 2 c.c. of concentrated hydrochloric acid to 2 c.c. of the oxidized material, and heat to boiling. If the original sample contained as little as 1% of methyl alcohol, a bright violet coloration develops. Less peptone and less ferric chloride make it more sensitive.

5. To 2 c.c. of the material add 1 c.c. of concentrated sulphuric acid, cool, and add 5 c.c. of fuchsin bisulphite solution; mix and allow to stand. The presence of formaldehyde is indicated by the violet red color which gradually develops.

The *fuchsin solution* is made as follows:

Dissolve 0.5 gm. of basic fuchsin in 200 c.c. of distilled water; add an aqueous solution of sulphur dioxide, the quantity corresponding to 1 gm. of sulphur dioxide gas; allow to stand until the solution assumes an amber color (this requires about one hour). The colorless fuchsin bisulphite solution which is obtained when more than 1 gm. of sulphur dioxide is added to 0.5 gm. of fuchsin is valueless as a reagent after standing two days. A solution prepared as outlined will keep well for ten days, although it is recommended not to use a solution after seven days.

6. Place 10 c.c. of the oxidized material in a small evaporating dish, and add 10 c.c. of strong ammonia. Evaporate just to dryness on a water bath. The formaldehyde con-

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[1] Gettler: J. Biol. Chem. 43: 2, June, 1920.

denses with the ammonia to hexamethylenetetramine. Dissolve this latter in a few drops of water. Add a drop of 5% mercuric chloride solution to a small amount of this solution, and place on a microscopic slide. Typical crystals of hexamethylenetetramine-mercuric chloride develop. The shape of these crystals varies with the concentration. It is, therefore, best to make control experiments for comparison with the suspected material.

7. Place in a test tube 3 c.c. of the oxidized distillate, 0.04 to 0.06 gm. of β-naphthol, and 3 to 5 drops of concentrated hydrochloric acid. Boil gently until the liquid becomes filled with an abundant precipitate of small white needles. Filter while hot. Wash with 1 c.c. of dilute alcohol (1:2). Boil the precipitate with 4 c.c. of dilute alcohol (1:1). (It is not necessary that all should dissolve.) Cool and filter off the precipitate. Wash with 1 c.c. of dilute alcohol (1:1). Dry on porous tile in a warm place and determine the melting point.

Methylene-di-β-naphthol, the product, forms white needles which, when the temperature in the neighborhood of the melting point is raised at the rate of 1° in fifteen seconds, begins to turn brown at 180°. It melts with decomposition to a red brown liquid at 189°-192° (uncorrected).

TOLUENE POISONING

Von Oettingen, Neal, and Donahue[1] showed that the concentration of toluene in the blood and elimination of hippuric acid in the urine were tests to determine toluene poisoning. Elimination of hippuric acid in the urine and concentration of toluene in the blood increase with the concentration of toluene in air. They found, in contrast to experience with benzene, the ratio of inorganic sulphates in the urine was not noticeably affected by exposure to toluene.

For the determination of the hippuric acid in the urine, they used Quick's method in the modification described by Kraus and Dulkins[2] in 1941.

To make this determination, acidulate the urine with acetic acid until acid to litmus and then concentrate to a volume of 150 c.c. by evaporation. To this concentrated urine, add sodium chloride, while heating and stirring, until a total of 45 gm. have been added (30 gm. per 100 c.c.). Cool to room temperature and, while stirring and scratching the sides of the beaker, add sulphuric acid of the concentration 1:1, drop by drop, until no further precipitate of hippuric acid is formed. Usually 2 to 5 or 6 c.c. of sulphuric acid are sufficient. In order to insure complete precipitation, add additional sulphuric acid to the filtrate, and in case no further precipitation occurs, allow the precipitate to stand for at least 15 minutes and then filter. Wash precipitate and beaker repeatedly with a 30% solution of sodium chloride which is free of sulphate ions, until the washings give a negative test for sulphate ions. When the washing is completed, transfer precipitate and filter to the beaker in which the original precipitation was made, add 75 to 100 c.c. of distilled water, and heat the mixture until the precipitate is dissolved. Titrate, hot, with 0.5 normal sodium hydroxide with phenolphthalein as indicator, and from the amount of sodium hydroxide used, calculate the quantity of hippuric acid. Whereas with concentrations of more than 300 mg. of hippuric acid per 150 c.c. of urine this method gives fairly accurate results, these become increasingly less exact with lower concentrations because comparatively large quantities escape the precipitation.

With this procedure, the normal hippuric acid excretion in the urine within 24 hours was determined as 403 mg. (average of 6 persons), which is somewhat lower than that frequently quoted in the literature, namely, 0.7 gm. These determinations show that the amount of hippuric acid excreted increased, roughly, with the intensity of the exposure.

In addition, the determination of toluene in blood may be utilized for appraisal of the intensity of the exposure. From the cubital vein of two of the subjects, von Oettingen and associates withdrew 5 c.c. of blood at the end of the exposure and the toluene content was determined with the method of Yant, Pearce, and Schrenk.[3] This was modified in several respects in their laboratory. Their results show that the concentration of toluene in blood increases as its concentration in air increases. In order to further prove this point, three dogs were exposed experimentally simultaneously with the human subjects to concentrations of 300 parts per million of toluene in air, and the concentration of toluene in their blood was determined at the end of the experiment. This was found in two animals to be 0.74 and 0.79 mg. per 100 c.c. of blood, and in the third, in which the sample was smaller than usual, 1.04 mg. per 100 c.c. These first two values check very well with the 0.73 mg. per 100 c.c. which was found in the human beings in the same experiment. It appears, therefore, that dogs can be well utilized for such determinations. It was found that concentrations of around 0.5 mg. per 100 c.c. of blood may already have caused definite impairment of certain nervous functions.

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[1] J. A. M. A. **118**: 579, 1942.
[2] J. Lab. & Clin. Med. **26**: 729, 1941.

DETECTION OF CYANIDE IN TISSUES

The cyanide odor often suggests the presence of cyanide. However, a chemical and biological test must also be made.

Detection of Cyanide in Human Tissues.[4]—The following tests have been recommended for the detection of cyanide in gastrointestinal contents, body tissues and fluids:

1. **Copper-Guaiac Test.**—Place about 50 gm. of finely ground organ or a few centimeters of stomach contents in a 100 c.c. flask. Acidify the material with tartaric acid, and suspend above it a strip of freshly prepared copper sulphate-guaiac paper (filter paper which has been wet in turn with 10% alcoholic solution of guaiac and 0.1% aqueous copper sulphate solution), stopper the flask, warm contents a little and allow to stand about one-half hour. If a blue or green color does not develop on the paper then cyanide is definitely excluded. No further tests for cyanide need be done, because this is the most sensitive test available. If a blue color develops, cyanide may be present. Since, however, the blue color is also produced by ammonia gas, hydrogen, chloride, ozone, oxides of nitrogen, bromine, chlorine, and hydrogen peroxide, 2 or 3 of the following tests must be resorted to in order definitely to establish the presence of cyanide in the material.

For the following tests, the distillates obtained by steam distillation of the acidified organs and stomach contents are used. See page 2243.

2. **Liebig's Thiocyanate Test.**—Place 20 c.c. of the distillate and 2 c.c. of ammonium polysulphide in an evaporating dish. Evaporate to dryness on a steam bath. Treat the residue with 5 c.c. of water, slightly acidify with hydrochloric acid, warm it a little, stir thoroughly, and allow to stand several hours so that the colloidal sulphur may precipitate. Filter. Add 10% ferric chloride dropwise until a maximum color intensity is obtained. A red color indicates the presence of cyanide.

A variation of the test is: Boil an aliquot portion of the distillate with ammonium sulphide for a few minutes, concentrate to 1 c.c., acidify with hydrochloric acid, and extract several times with ether. Allow the ethereal extracts to evaporate spontaneously. Add ferric chloride solution dropwise to the residue as long as the color deepens. If a red color develops, which is soluble in ether, the presence of cyanide is established.

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[3] Yant, W. P., Pearce, S. J., and Schrenk, H. H.: A Microcolorimetric Method for the Determination of Toluene. Washington, D. C., Bureau of Mines Reports, Investigation, 3323, 1936.

[4] Gettler, A. O. and Baine, J. O.: Am. J. M. Sc. **195**: 182, 1938.

3. **Prussian Blue Test.**—Make 20 c.c. of the distillate strongly alkaline with sodium hydroxide solution, and concentrate to about 2 c.c. Add 2 drops of 5% ferrous sulphate and 1 drop of 10% ferric chloride. Warm the mixture slightly. Add concentrated hydrochloric acid drop by drop, avoiding an excess, until the brown precipitate of iron hydroxide just dissolves. If cyanide is present, a deep blue color or precipitate of Prussian blue remains, which is insoluble in dilute hydrochloric acid.

4. **Vortman Test.**—To 5 c.c. of the distillate add a few drops of 10% potassium nitrite solution and 2 to 4 drops of 5% ferric chloride, and sufficient dilute sulphuric acid to give the mixture a bright yellow color. Heat the mixture to boiling, allow to cool, add sufficient ammonia to precipitate the excess of iron, and filter. To the filtrate add a few drops of very dilute ammonium sulphide solution. If cyanide is present, a violet color appears which gradually passes through blue, to green, and then yellow.

5. **Phenolphthalein Test.**—To 2 c.c. of the distillate add 2 or 3 drops of a colorless alkaline solution of phenolphthalein (made by boiling, under a reflux, a dilute sodium hydroxide solution of phenolphthalein with zinc dust until colorless) and a drop of very dilute (1 to 2,000) copper sulphate solution. The development of a red color indicates the presence of cyanide.

6. **Picric Acid Test.**—Make 5 c.c. of the distillate faintly alkaline with sodium hydroxide solution, and add 2 or 3 drops of saturated picric acid. On warming, a red color develops if cyanide is present in the sample.

7. **Ferrous-Uranic Test.**—Dissolve about 5 mg. of ferrous sulphate, and the same quantity of uranic nitrate in 50 c.c. of water. Place 1 c.c. in a porcelain dish and add 1 or 2 drops of the distillate to be tested. The appearance of a gray-purple color or purple precipitate indicates the presence of cyanide.

8. **Silver Test.**—Place 30 c.c. or less of the distillate in a 50 c.c. flask, and acidify with 6N nitric acid. Place a hanging drop of silver nitrate solution on a glass slide over the mouth of the flask. After 30 minutes, carefully remove the slide, add 1 drop of dilute nitric acid to the drop on the slide, and place a cover slip over it. Slightly heat the material under the cover slip by playing a very small flame across the underside of the glass slide. On cooling, long slender crystals of silver cyanide are visible under the microscope. Silver chloride crystallizes as octahedra, and can therefore easily be differentiated from silver cyanide.

Test No. 1 is used as a preliminary rapid and sensitive method of ruling out the presence of cyanide. For definitely establishing its presence, Tests 2, 3, and 8 are recommended.

Gettler and Baine Method for Cyanide

The following analytical methods for the detection of cyanide in tissues are those of Gettler and Baine.[1]

Immediately upon removal of the organs from the body, place them in a covered receptacle in the refrigerator. When ice cold, pass 125 gm. through a meat grinder previously cooled by cracked ice. These precautions are to prevent any loss of HCN by volatilization. Quickly place 100 gm. of the finely divided tissue in a 500 c.c. flask (Fig. 688, *B*), and add 300 c.c. of cold water, 2 c.c. white mineral oil, and 10 c.c. of 5% tartaric acid. Mineral oil prevents frothing during distillation; tartaric acid facilitates complete removal of the cyanide. Connect the flask to a vertical, water-cooled condenser (*C*), and join this to 3 absorption tubes (*D*), connected in series. Each absorption tube contains 10 c.c. of N/10 sodium hydroxide. Apply suction to the side-arm of the last absorption tube. Heat the water in bath (*A*) to boiling, then regulate the suction so that the content of the flask (*B*) is vigorously agitated, but slowly (about 5 drops per minute) distills into the first alkali absorption tube. Any escaping HCN is absorbed from tube D_1 in the alkali solution of D_2 and D_3. After 2 hours of distillation, shut off the suction and disconnect from the distillation flask at *E*. Transfer the 3 alkali absorption solutions quantitatively to a 100 c.c. volumetric flask, and make up to the 100 c.c. mark. If very small quantities of cyanide are suspected in the tissues, keep the final volume down to 50 c.c. by washing each tube successively with the same wash water. If the resulting solution is not perfectly clear, cool to 0° C., and then filter through a dry filter paper. Take aliquot portions for analysis.

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[1] Am. J. M. Sc. **195**: 182, 1938.

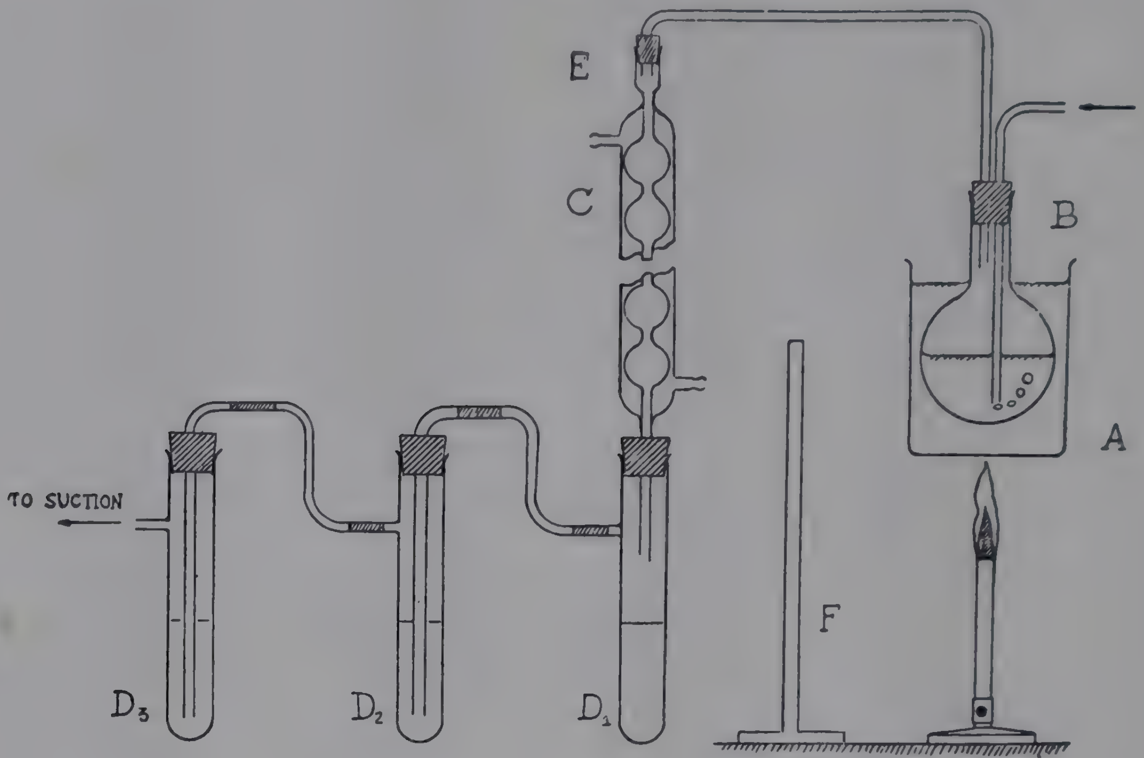

Fig. 688.—*A*, Water bath; *B*, round-bottom flask; *C*, reflux condenser; D_1, D_2, D_3, absorption tubes; *F*, asbestos screen. (Gettler and Baine: Am. J. M. Sc., 1938.)

Gettler and Baine Quantitative Determination of Cyanide

For the quantitative estimation of cyanide, Gettler and Baine tested out the various methods and found the Colorimetric Ferric Thiocyanate Method and the Volumetric Silver Nitrate Method fairly accurate. These are as follows:

9. **Colorimetric Ferric Thiocyanate Method.**—Place in a small evaporating dish an accurately measured aliquot of the alkaline absorbed distillate, containing in the neighborhood of 1 mg. of HCN (as previously determined by the picrate method), and add 10 c.c. of ammonium polysulphide.* Evaporate the mixture to dryness on the water bath. Add to the residue 30 c.c. water and N/10 hydrochloric acid until distinctly acid. Heat slightly, stir thoroughly, and allow to stand overnight to insure complete precipitation of the colloidal sulphur. Filter into a 50 c.c. volumetric flask, washing the evaporating dish and residue on filter paper with small portions of water, and adding washings to the main filtrate. Add 5 c.c. of 10% ferric chloride and enough water to bring the volume to the 50 c.c. mark, mix thoroughly, and compare the red color obtained in a colorimeter with a standard potassium thiocyanate solution (3.6 mg. potassium thiocyanate, equivalent to 1 mg. HCN, plus 5 c.c. of 10% ferric chloride made up to 50 c.c. with water). Should the HCN content in the distillate be very small (0.2 mg. or less in 10 c.c.) then reduce all working volumes and keep the final volume down to 25 c.c.

10. **Volumetric Silver Nitrate Method.**—Place 25 c.c. of the alkaline absorbed distillate containing the cyanide in a 50 c.c. Erlenmeyer flask. Add 2 c.c. concentrated ammonium hydroxide and 1 c.c. of 10% potassium iodide solution. Titrate the cyanide with 0.005/N

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*Saturate 600 c.c. of concentrated ammonium hydroxide with H_2S, then dilute to 1 liter with concentrated ammonium hydroxide. Add an excess of powdered sulphur, and let stand, shaking occasionally, until saturated.

silver nitrate solution. The end point occurs with the formation of a bluish-white opalescent cloud of silver iodide, which is very sharp, and most easily observed against a black background.

Calculation: each c.c. of 0.005/N silver nitrate solution is equivalent to 0.266 mg. of HCN.

11. **Biological Presumptive Test for Cyanide.—**

Take up a portion of the material to be tested in a dropper with a rubber bulb.

Feed it to a guinea pig.

If the guinea pig fails to show symptoms within five minute, feed it a small amount more of the specimen.

If 15 c.c. fail to give symptoms, cyanide is not present.

If, upon feeding, the guinea pig dies with typical cyanide convulsions, within one to ten minutes, the specimen probably contains cyanide.

12. **Other Simple Chemical Tests.—**

Grind up a portion of the material to be tested.
Cover it with distilled water.
Distill the specimen over steam.
Collect several portions of the distillate.
Test as follows:

1. Mix equal parts of the distillate and yellow ammonium sulphide.
Evaporate to dryness.
Add a few drops of hydrochloric acid, c. p., and 5 c.c. of 10% ferric chloride.
A blood red color develops if cyanide is present.

2. Mix equal parts of the distillate and 10% ferric chloride and ferrous sulphate.
Add a few c.c. 10% potassium hydroxide.
A green precipitate is formed.
Add hydrochloric acid.
If cyanide is present, a Prussian blue is formed.

3. Place about 5 c.c. of the distillate in a test tube.
Add 2 c.c. 5% silver nitrate solution.
A white precipitate is formed if cyanide is present.

Is Cyanide Normally Present in Human Organs?—

Gettler and Baine failed to detect HCN in normal tissues.

What is the minimum lethal absorbed dose of cyanide? 0.5 mg. HCN per kilo of body weight.

What is the effect of putrefaction on the cyanide content of tissues? Putrefaction interferes in the colorimetric picrate method and in the silver nitrate titration. The colorimetric thiocyanate method alone gives reliable results when applied to putrefied material. Cyanide material is little changed during putrefaction.

Is cyanide produced during putrefaction of normal tissue? A small amount of cyanide is produced during putrefaction, 0.01 to 0.03 mg. of HCN per 100 gm. of tissue, but since this is only one-tenth of the lowest content ever found in organs where death was due to cyanide poisoning, putrefaction should in no way interfere in deciding a cyanide poisoning case.

Does embalming alter the cyanide content of organs? Cyanide in tissue is rapidly lost when the tissue is embalmed with formaldehyde.

MICROCHEMICAL DETERMINATION OF YELLOW PHOSPHORUS[1]

The following method for the confirmation and quantitative determination of yellow phosphorus uses a spot test color reaction which is confined to a standard area, the color intensity of which is proportional to the concentration of phosphorus present. This method is not only specific and sensitive, but also very simple. The test is based upon the formation of phosphine, which in turn easily reduces a mercuric bromide test paper, yielding a canary yellow color.

Phosphorus may exist in several forms; the best known of these are yellow and red phosphorus. Red phosphorus is considered by most authorities as nontoxic, whereas yellow phosphorus is very toxic, 0.1 gram having been recorded as a fatal quantity. Yellow phosphorus is used commonly in rat poison pastes. Acute poisoning is usually accidental, although there are records of suicides and homicides in which phosphorus had been used. The method outlined here is applicable to yellow phosphorus only, and is satisfactory from a toxicologic standpoint.

Phosphorus may remain in the body for several days; the greater the delay in obtaining the specimen, however, the less the possibility of detecting phosphorus. The procedure of isolating it for analysis depends upon its volatility. Stomach contents are best suited for analysis. The liver is the organ of choice.

Symptoms.—Progressive pain and burning sensations along the gastrointestinal tract, eructation of gas, choking, vomiting, and diarrhea are symptoms of phosphorus poisoning. Other symptoms are frequently noted: garlic odor on the breath, jaundiced skin, bleeding from the nose and from the intestines, muscular paralysis, and impairment of the special senses. Terminally, stupor, delirium, and coma occur. Death ensues usually within two to six days.

Treatment.—Irrigate the stomach with 1 per cent potassium permanganate solution and large amounts of suspended charcoal. If this is done quickly, the treatment may be effective.

Post-Mortem Findings.—There is erosion of the mucous membrane of the gastrointestinal tract, with fatty degeneration and acute yellow atrophy of the liver. The stomach contents, as a rule, have the odor of garlic, and if viewed in the dark, give off a greenish phosphorescent light.

Chemical Analysis.—The following *qualitative* tests have been used to identify phosphorus:

1. **Odor.**—A garlic-like odor may be recognized upon opening the stomach.

2. **Scherer's Test.**—Have on hand Whatman No. 40 filter paper cut in strips, and impregnated with silver nitrate, and other strips impregnated with lead acetate.

Place a portion of the finely divided material to be examined in an Erlenmeyer flask. Suspend it in distilled water.

Suspend 2 strips of filter paper over the suspected sample, one impregnated with silver nitrate and the other with lead acetate.

Heat the contents at about 50° C. on a water bath, for approximately one hour.

If the silver nitrate paper turns black, either phosphorus or hydrogen sulphide may be present.

If both papers turn black, hydrogen sulphide is present, and phosphorus may also be present.

The fact that the silver nitrate paper turns black is not conclusive proof that phosphorus is present, since any volatile reducing agent, such as formaldehyde, may also give the same reaction. This test, therefore, is more important in indicating the absence of phosphorus rather than its presence.

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[1] Kaye, Sidney: J. Lab. & Clin. Med. 28: 2, Nov., 1942.

Quantitative Test for Phosphorus (Kaye)

Set up a distillation apparatus as illustrated in Fig. 689.

Transfer a weighed aliquot of the sample to a steam distillation apparatus containing about 100 c.c. of distilled water. Use an ice-cooled receiver to collect the distillate.

Shield the light from the Bunsen burner by a sheet of asbestos to prevent interference in the visual detection of the phosphorescence emitted.

Carry out the distillation in the dark.

Collect the distillate in a small volume of 0.5 N silver nitrate, cooled in a 125 c.c. Erlenmeyer flask immersed in an ice bath.

Greenish, phosphorescence may be seen as the phosphorus vapors reach the condenser (Mitscherlich's phosphorescence test). As little as 60 gamma[2] in 200 grams of material will give a good phosphorescence. However, in the presence of alcohol, ether, benzene, turpentine, phenols, chloroform, essential oils, oxidizing agents, mercury, copper, and silver, there may be a marked diminution or complete absence of phosphorescence.

Continue distillation until about 75 c.c. are collected. As the first traces of phosphorus come over into the silver nitrate solution, a black precipitate of silver phosphide forms. After about 75 c.c. of distillate have been collected, no further precipitate will form.

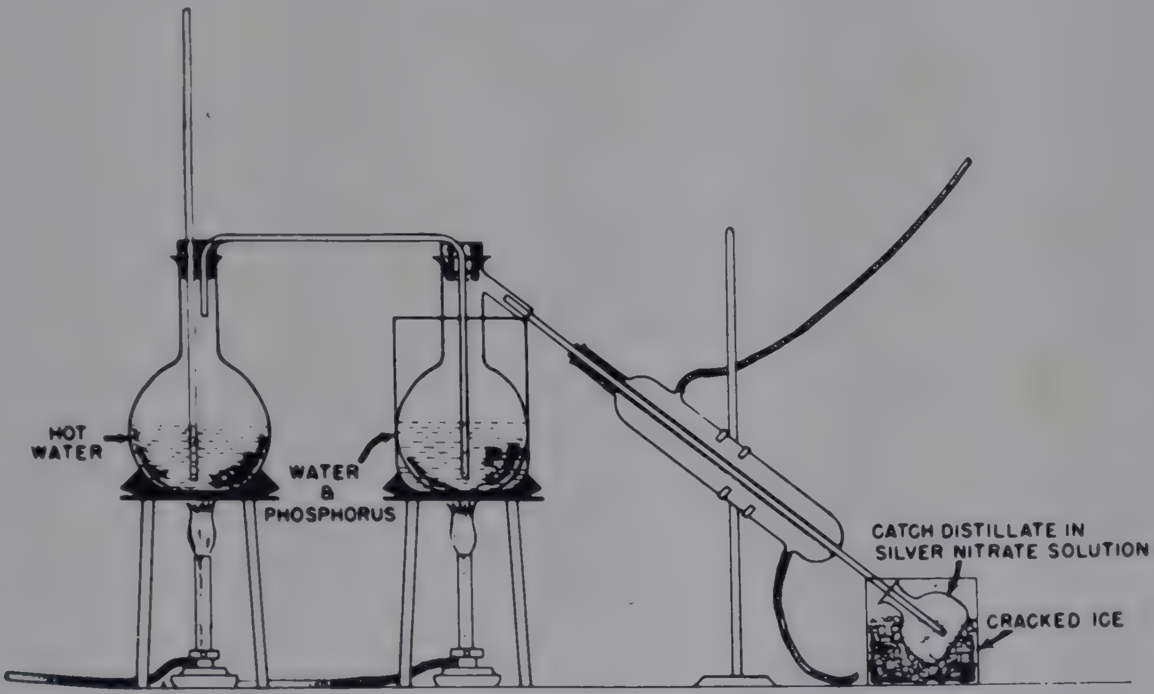

Fig. 689.—Distillation apparatus for phosphorus determination, method of Kaye.

Silver phosphide is a heavy precipitate and settles rapidly. Siphon off the supernatant liquid without disturbing the precipitate, leaving a small amount of the liquid behind.

Wash the precipitate in the flask with several portions of distilled water to remove the last traces of any silver nitrate which might have adhered to the precipitate.

Again siphon off the supernatant fluid. Two washings usually suffice.

Test the washings for silver ions by using a chloride solution.

If the precipitate in the receiving vessel is slight, make up the volume to 50 c.c. with distilled water.

If the precipitate is heavy, dilute to a larger definite volume with distilled water.

Swirl the solution well so as to divide the precipitate evenly, withdraw a small aliquot, and record the amount withdrawn.

Place this aliquot in a previously prepared modified Gutzeit apparatus. See Fig. 690.

Modified Gutzeit Apparatus.—The modified Gutzeit apparatus consists of a 125 c.c. Erlenmeyer flask containing some arsenic-free zinc, several drops of 5 per cent copper sulphate solution to activate the generating process, and 25 to 40 c.c. of 20 per cent sulphuric acid. The amount of sulphuric acid should be sufficient to dissolve the precipitate in the aliquot which is to be added. Excess does not interfere. The stopper in the flask supports a small calcium chloride drying tube into which is plugged some cotton impregnated with lead acetate. Above this tube is attached a pair of flanges[3] between which is placed a disk of mercuric bromide sensitized filter paper. When the silver phosphide comes in contact with the hydrogen generating solution, phosphine is liberated.

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[2] One gamma = 0.000001 gram or 0.001 mg.

Keep the generating flask warm in a water bath at about 50° C. Phosphine travels upward and is purified from hydrogen sulphide by passage through the lead acetate-saturated cotton. On reaching the flanges, where it comes in contact with the moistened mercuric bromide paper, a yellow color results which is directly proportional to the phosphine produced.

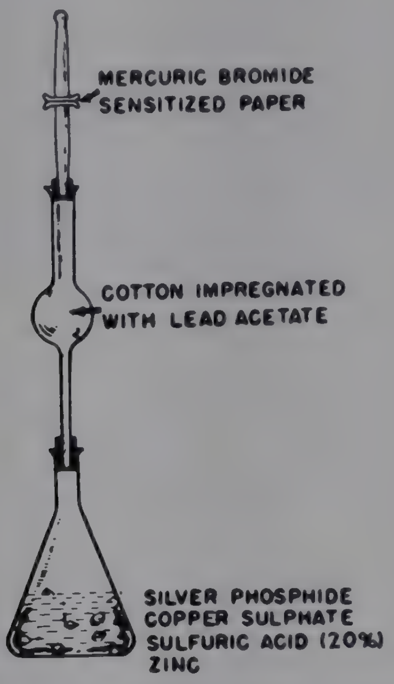
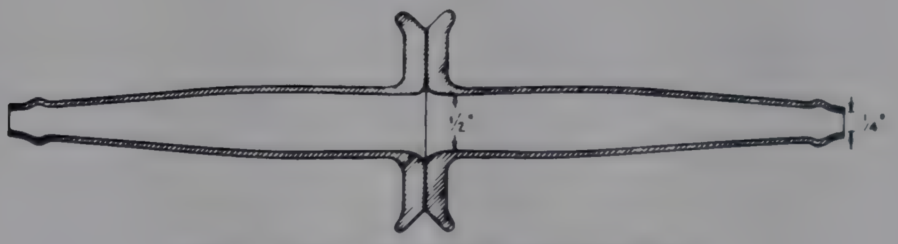
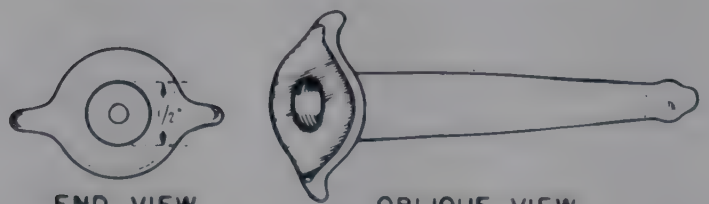


Fig. 690.—Modified Gutzeit apparatus for the determination of phosphorus.



SIDE VIEW (TWO UNITS ASSEMBLED)



END VIEW (SINGLE UNIT)

OBLIQUE VIEW (SINGLE UNIT)

Fig. 691.—Microchemical flanges for the modified Gutzeit apparatus.

Since the bores of the several flanges are uniform, comparisons can be made with phosphorus standards prepared in a similar manner. These yellow standards are fairly stable and can be kept for several months once they have dried.

This color reaction is sensitive to less than 10 gammas of phosphorus.

CAUTION: To make sure that the zinc and other reagents are arsenic and phosphorus free, before adding the silver phosphide, liberate hydrogen for some time and then examine the filter disk. There should be no discoloration of the filter disk. Add the silver phosphide and put the flanges in place. Allow the generator to run for about half an hour.

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[3] Flanges may be purchased from Eck and Krebbs, or from E. Machlett & Sons, both of New York.

Microchemical Flanges

The microchemical flange used for this determination is illustrated in Fig. 691. These flanges are of great value in quantitative gas and solution analyses where the intensity of a color reaction, which is proportionate to known amounts of the substance concerned, can be recorded.

They have been used in the determination of sulphides, cyanides, arsenic, antimony, carbon monoxide, and other substances. For substances in solution, which give brilliant graded colors with a detector, the flanges have also proved successful.

The flange device is used in the research Laboratories of the St. Louis Police Department, Toxicology Laboratories in New York City, Baltimore, Washington, D. C., and Virginia.

Mercuric Bromide Papers

Mercuric bromide sensitized paper is prepared by cutting disks of Whatman No. 40 filter paper which are then impregnated with 5 per cent mercuric bromide in 95 per cent alcohol for about 5 minutes. After shaking off the droplets, a disk is fastened between the flanges with a rubber band.

TEST FOR DETERMINING CHLOROFORM CONTENT OF THE BRAIN FOLLOWING ANESTHESIA

The problem of the determination of the chloroform content of the brain following anesthesia is sometimes of medicolegal interest. Gettler and Blume[1] described the technic which they have worked out for this determination. It has been shown that a brain of a person sufficiently anesthetized for a minor operation contains 120 mg. to 182 mg. of chloroform in 1,000 gm. of brain tissue. Gettler and Blume made intensive investigations on dogs which were killed by excessive administration of chloroform and then tested. Animals when fully anesthetized with chloroform showed in the brain 270 mg. and 284.6 mg. in 1,000 gm. of tissue. An animal in the stage of recovery showed a drop in the amount of chloroform in the brain during the first thirty-four minutes, so that only 51.3 mg. in 1,000 gm. of brain tissue were left. From this time on, the chloroform content decreased more slowly, and after 190 minutes there was only 0.16 mg. of chloroform present in 1,000 gm. of brain tissue. In the stage of recovery, the lungs contained much less chloroform than the brain.

Gettler and Blume's technic for their experimental work on animals is given below and can well be followed in dealing with human material.

Ten dogs were used in these experiments. After being tied to the animal board, they were anesthetized with chloroform, the open mask method being used. They were kept in a state of surgical anesthesia for definite periods. The mask was then removed, and the animals were allowed to recover spontaneously. Dog 6 was not allowed to recover but was sacrificed by excessive administration of chloroform. In dogs 5 and 7, the trachea was clamped while they were fully anesthetized. In order to investigate the chloroform content of the brain at various stages of recovery, the dogs were sacrificed at stated intervals. Painless death was obtained by injecting morphine and then exposing and clamping the trachea. Autopsy was then performed. The brain and lungs were removed, weighed and placed in the ice box in sealed containers until the analysis was started.

Method of Analysis.—The brain and lungs, when ice cold, were ground up, and quickly placed in a 500 c.c. distillation flask; about 100 c.c. of water was added and also a little tartaric acid, enough to make the mixture acid in reaction. The material was then subjected to steam distillation, a long, well-cooled condenser and an adapter being used. The receptacle for collecting the distillate, a 300 c.c. Erlenmeyer flask, contained about 10 c.c. of ice cold, acidified hydrochloric acid water and was packed in ice. The tip of the adapter reached into the ice cold water contained in the receptacle for distillate. With these precautions, there was no loss of chloroform during distillation. Our experiments have proved that, if 250 c.c. of distillate are collected, all of the chloroform in the tissues will have passed into the distillate. The distillate was well mixed, measured, stoppered, and kept cold; 5 c.c. portions of this distillate were taken for the colorimetric determination.

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[1] Arch. Path., p. 841, June, 1931.

Colorimetric Estimation of Chloroform in Distillate.—The materials necessary for the colorimetric estimation of the chloroform in the distillate were: chemically pure pyridine (colorless), 20% sodium hydroxide solution, and the standards to be described.

Chloroform Standards.—One gram of pure chloroform was weighed by placing slightly more than 1 gm. of chloroform in a weighing bottle with a groundglass stopper, allowing the chloroform to evaporate spontaneously and weighing from time to time (with weighing bottle stoppered) until the weight of the chloroform in the bottle was just 1 gm. This gram of chloroform was then dissolved in about 900 c.c. of water contained in a 1-liter volumetric flask; 5 c.c. of hydrochloric acid were added, and finally enough water was added to bring the total volume to exactly 1 liter. The whole was thoroughly mixed. Precautions were taken to avoid evaporation of the chloroform during the transfer. This solution contained 1 mg. of chloroform in each cubic centimeter. From this stock solution, a series of weaker standards were made, having the following chloroform values: 0.5 mg. in 1 c.c.; 0.1 mg. in 1 c.c.; 0.01 mg. in 1 c.c.; 0.005 mg. in 1 c.c.; 0.0025 mg. in 1 c.c. These standards do not keep; new standards were made for each set of determinations.

Procedure.—Into five clean, dry, 50 c.c. test tubes were pipetted 5 c.c. portions of the five standards containing, respectively, 0.5 mg., 0.1 mg., 0.01 mg., 0.005 mg. and 0.0025 mg. of chloroform per cubic centimeter (see Preparation of Standards). Into a similar test tube were pipetted 5 c.c. of the distillate. To each of the tubes, 5 c.c. of chemically pure (colorless) pyridine and 10 c.c. of 20% sodium hydroxide solution were added. The contents of each tube were mixed and the tube loosely corked to avoid evaporation. The tubes were placed in a boiling water-bath for exactly one minute and then quickly cooled by running water; 20 c.c. of water were added to each tube, and the contents were mixed. Of the series of standards, the one that matched the color of the unknown closest was used as the standard and read against the unknown in the colorimeter.[1]

Calculation.—

$$\frac{\text{Standard}}{\text{Unknown}} \times \begin{matrix}\text{Value of standard used in}\\ \text{milligrams of chloroform}\end{matrix} \times \frac{250}{5} \times 1.087 \left(\begin{matrix}\text{correction for per-}\\ \text{centage of recovery}\end{matrix}\right)$$

$$= \text{milligrams of chloroform in grams of tissue used.}$$

DETECTION OF CHLOROFORM IN THE BRAIN, LUNGS, AND LIVER

The detection of chloroform in the organs of cadavers has been reported by a number of observers. Fischer[2] found 55 mg. in 780 gm. of lungs and blood in complete narcosis; 70 mg. in 480 gm. of brain; a trace in 445 gm. of liver, kidney, and spleen. Sedya,[3] in a case of beginning narcosis, found 4 mg. in the brain, 7.2 mg. in 1,000 gm. of stomach and contents; 2 mg. in liver, kidney, and spleen; 4.2 mg. in lungs, heart, and blood. Tissot[4] found 450 mg. in 1,500 gm. of brain; 350 mg. in 1,500 gm. of liver; 0.023 per cent in muscle (230 mg. in 1,000 gm.). Nicloux[5] found 0.0555 per cent in brain (555 mg. in

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[1] Gettler, O. A., and Blume, Hyman: Arch. Path. **11**: 554, 1931.

[2] Jahresb. d. chem. Untersuchungsamtes d. Stadt Breslau, f. d. Zeit April 1, 1894—March 31. 1895.

[3] Lesser Vierteljahrsschr. f. gerichtl. Med., 1899.

[4] J. de physiol. et de path. gen. **8**: 423, 1906.

[5] Compt. rend. Soc. de biol. **60**: 248. 1906.

1,000 gm.); 0.05 per cent in liver; 0.046 per cent in kidney; 0.038 per cent in spleen; 0.021 per cent in muscle; 0.07 per cent in arterial blood; 0.04 per cent in heart; 0.083 per cent in spinal cord. Results of other workers in this field show similar deviations.

Technic for this determination has given such uncertain results that Gettler and Blume suggested the following method, which is a modification of the colorimetric method described by Cole,[6] and is based on the Fujiwara reaction. The Fujiwara[7] reaction* has a pyridine color reaction with chloroform.

1. Use 5 c.c. of distillate for the colorimetric determination. Since the total distillate is 250 c.c., the actual value found in 5 c.c. is multiplied by 50 and not by 250, as is the case when 1 c.c. of distillate is used. This makes the method more accurate.

2. After the color has developed, there are present two layers; the upper pyridine layer contains the developed pink color. Cole pipettes this off into a colorimeter cup. The disagreeable, poisonous vapor of the pyridine makes this objectionable. It was found that if exactly 20 c.c. of water are added and mixed, the two layers merge into one, the entire mixture taking on the pink color; no turbidity, even in weak chloroform solutions, is then encountered. This modification reduces the sensitivity to 1:100,000 which is quite adequate for most toxicologic work.

3. Colored-glass disks were devised to be used as permanent standards in the Hellige-Klett colorimeter (comparative type).

With these modifications, the method used by Gettler and Blume is in detail as follows:

Method.—

Isolation of Chloroform From Tissues.—As soon as the brain, lungs, and liver are removed from the body, place in a clean jar, seal air-tight, and place in a refrigerator. When the material is ice cold, grind about 150 gm. of the tissue, weigh 100 gm. of the minced tissue, and quickly place it in a 500 c.c. distillation flask. Add about 100 c.c. of water and enough tartaric acid to make the mixture acid. Subject to steam distillation (Fig. 682), using a long, well-cooled condenser and an adapter. Collect the distillate in a 300 c.c. Erlenmeyer flask containing about 10 c.c. of ice cold, acidified hydrochloric acid water, keeping the collecting flask packed in ice. The tip of the adapter should reach into this ice cold water contained in the distillate receptacle. With these precautions there should be no loss of chloroform during distillation. Experiments have proved that if 250 c.c. of distillate are collected, all of the chloroform originally present in the tissue will have passed into the distillate. The distillate is well mixed, measured, stoppered, and kept cold. For the colorimetric determination, 5 c.c. portions of the distillate are taken.

Preparation of Standards for Use in Colorimetric Estimation of Chloroform in Distillate.—$R\text{-}C\text{-}halogen_3$ compounds, such as bromoform, carbon tetrachloride, chloretone, chloral, and iodoform, also give the Fujiwara color reaction. It is therefore essential at the outset to determine whether chloroform or some other one of the aforementioned group is present.

The materials necessary for the colorimetric determination of chloroform in tissues are: chemically pure pyridine (colorless), 20% sodium hydroxide solution, and standards such as are described in the following paragraphs:

Chloroform Standards: Place slightly more than 1 gm. of chloroform in a weighing bottle having a ground-glass stopper. Allow it to evaporate spontaneously, weighing from time to time, with the weighing bottle stoppered, until the weight of chloroform in the bottle is exactly 1 gm. Dissolve this 1 gm. in about 900 c.c. of water contained in a

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*See p. 2275.

[6] J. Biol. Chem. 71: 173, 1926.

[7] Stizungsb. u. Abandl. naturfors. Geselsch. Rastock 6: 1, 1914.

1-liter volumetric flask. Add 5 c.c. of hydrochloric acid and enough water to bring the total volume to exactly 1 liter. Mix thoroughly. Take precautions to avoid evaporation of the chloroform during the transfer. The solution contains 1 mg. of chloroform in 1 c.c. Make a series of weaker standards from this stock, having the following chloroform values: 0.5, 0.1, 0.01, 0.005, and 0.0025 mg., respectively, per c.c. Keep in a refrigerator. These standards are then good for 1 week.

Permanent colored-glass standards (an alternate standard) are prepared as follows: Select a series of glasses with varying depths of orange pink color, simulating the different depths of colors obtained by various concentrations of chloroform when subjected to the pyridine test. Cut small disks of these glasses and fit into the circular openings of the wheel of the Hellige-Klett colorimeter, comparative type. Standardize these colored disks by chloroform solutions of known concentrations. The color disks 1 to 8, respectively, have the following values: Chloroform in 5 c.c. of solution, 0.125 mg., 0.25 mg., 0.375 mg., 0.5 mg., 1 mg., 1.5 mg., 2 mg., 2.5 mg. These colored-glass standards keep indefinitely if kept in the dark.

Colorimetric Estimation of Chloroform in Distillate.—The colorimetric method, using the chloroform standards, is applied as follows: Into five clean, dry, 50 c.c. test tubes (Folin digestion tubes may be used), measure 5 c.c. portions of the 5 standards containing, respectively, 0.5, 0.1, 0.01, 0.005, and 0.0025 mg. of chloroform per c.c. Into a similar test tube, pipette 5 c.c. of the distillate. To each of the tubes add 5 c.c. of chemically pure, colorless pyridine, and then 10 c.c. of 20% sodium hydroxide. Mix the contents of the tubes thoroughly, and cork loosely to prevent evaporation. Place in a boiling water bath for exactly 1 minute and then cool quickly with running water. Add exactly 20 c.c. of water to each tube and mix. Compare the color of the unknown with the colors of the five standards. Should the unknown match the color of the standard containing 0.01 mg. in 1 c.c. (0.05 mg. in 5 c.c.), the chloroform content of the entire distillate (250 c.c.) would be 50 times 0.05 mg., or 2.5 mg. Since 100 gm. of tissue were used in obtaining the distillate and the recovery is 92% (see under heading "Percentage of Recovery"), there are 2.5 times 1.087, or 2.72 mg. of chloroform in the 100 gm. used for analysis. If none of the five standards exactly matches the color of the unknown, the latter is compared in a colorimeter with that one of the five standards which has a color depth closest to the unknown. From the readings on the colorimeter, the chloroform content in 100 gm. of tissue is calculated as follows:

$$\frac{\text{Standard}}{\text{Unknown}} \times \begin{matrix}\text{value of standard used}\\ \text{in mg. of chloroform}\end{matrix} \times \frac{250}{5} \times 1.087 \left(\begin{matrix}\text{correction for per-}\\ \text{centage of recovery}\end{matrix}\right)$$

$=$ mg. chloroform in 100 gm. tissue.

The colorimetric method, using colored-glass standards, is especially adapted where routine determinations of chloroform are being done over long periods. The standard glasses are permanent. This method eliminates the preparation of a series of chloroform standards for each and every chloroform determination. It is applied as follows: Into a clean, dry, test tube pipette 5 c.c. of the distillate; add 5 c.c. of chemically pure, colorless pyridine, and then 10 c.c. of 20% sodium hydroxide; mix; cork the test tube loosely to prevent evaporation. Place in a boiling water bath for exactly 1 minute and then cool quickly in running water. Now add exactly 20 c.c. of water and mix. Place in the cell of the Hellige-Klett colorimeter. Turn the wheel of the instrument and compare the color of each disk with that of the solution in the cell. The disk that exactly matches the color of the solution in the cell is noted. The value of this disk in terms of milligrams of chloroform is known (see previous paragraph on permanent colored-glass standards).

Calculation:

$$\begin{matrix}\text{Value of colored}\\ \text{disk in mg.}\\ \text{chloroform}\end{matrix} \times \frac{250}{5} \times 1.087 \left(\begin{matrix}\text{correction for}\\ \text{percentage of}\\ \text{recovery}\end{matrix}\right) = \begin{matrix}\text{correction for}\\ \text{100 gm. tissue.}\end{matrix}$$

The chloroform content of the brain, lungs, and liver after chloroform anesthesia, given in preparation for surgical operation, is as follows (Gettler and Blume): brain, from 120 to 182 mg., lungs, from 92 to 145 mg., and liver, from 65 to 88 mg., in 1,000 gm. They stated that after death the chloroform content of the tissues decreases daily. After forty-two days, only 18 per cent of the original amount present is recoverable.

TESTS FOR CHLOROFORM AND CHLORAL HYDRATE

Hoffman Test

Place 5 c.c. of the unknown in a test tube.
Add 1 c.c. of aniline
and 1 c.c. of 10% sodium hydroxide.
Heat gently.

A sharp distinctive skunk-like odor (isonitrile) is produced if chloroform or chloral hydrate is present.

Fujiwara Test

Place 5 c.c. of the unknown in a test tube.
Add 1 c.c. of pyridine
and 1 c.c. of 10% sodium hydroxide.
Heat on a water bath for approximately 5 to 10 minutes.

A pink or red color develops if chloroform or chloral hydrate is present. This test may be used for partial quantitative estimation. Prepare varying standards using the same technic as used for developing color in the unknown solution. Match the color of the unknown with the color of these standards.

DETECTION OF SMALL AMOUNTS OF CHLORAL IN THE PRESENCE OF CHLOROFORM AND FORMALIN EMBALMING FLUID

The detection of chloral is quite difficult, especially when it is mixed with Formalin embalming fluid. Formalin interferes with the color reaction of chloral in that it gives the same color, namely, a brownish red. The objection to the Schwartz method, which is commonly used, is that other substances besides chloral give the green fluorescence supposed to be characteristic of chloral alone. Gettler,[4] in an investigation of this test, observed that chloroform does not produce the green fluorescence, but that chloral does, and that the red color does not appear in very dilute solutions of chloral, although the green fluorescence is always present; further, he showed that it was sensitive to 0.01 mg. in 1 c.c.; that formaldehyde, acetaldehyde, formic acid, benzaldehyde do not give the reaction; he also found that if formaldehyde is present together with the chloral, the reaction is not interfered with provided the test is done at room temperature. Gettler's technic is as follows:

To 1 c.c. of distillate add 6 drops of saturated resorcin and 1 c.c. of saturated sodium carbonate solution or less if only traces of chloral are present. Let stand for one-half hour, then dilute by adding 10 c.c. of water. A beautiful green fluorescence results. In cases of extremely small quantities of chloral, viewing by direct sunlight against a black, glossy background, is of great advantage. Any development of color or fluorescence after many hours is of no consequence.

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[4] Proc. Soc. Exper. Biol. & Med. 16: 110, 1919.

ISOLATION AND IDENTIFICATION OF EASILY VOLATILE ORGANIC LIQUIDS IN HUMAN TISSUES

Method of Gettler and Siegel[5]

Among the organic liquids used in dry-cleaning, as general solvents, as fire-extinguishers, as anesthetics, and as therapeutic agents in hookworm disease are: ethyl chloride, ethylene chloride, propylene dichloride, carbon tetrachloride, dichlor-difluormethane, chloroform, carbon bisulphide, benzene, and diethyl ether. Fatalities have occurred from drinking some of these liquids or from inhaling their vapors. The layman, unaware of their toxicity, may inhale the vapors with impunity until unconsciousness and death result. Gradwohl in 1941 observed a near fatal case of carbon tetrachloride poisoning which bears out Gettler and Siegel's statement concerning the toxicity of the vapors of these liquids. This was the case of a man who went into his basement one night to extinguish a fire, using an extinguisher containing carbon tetrachloride. Severe and serious symptoms resulted, due unquestionably to the inhalation of vapors and to absorption from the thorough wetting of his clothing with this substance.

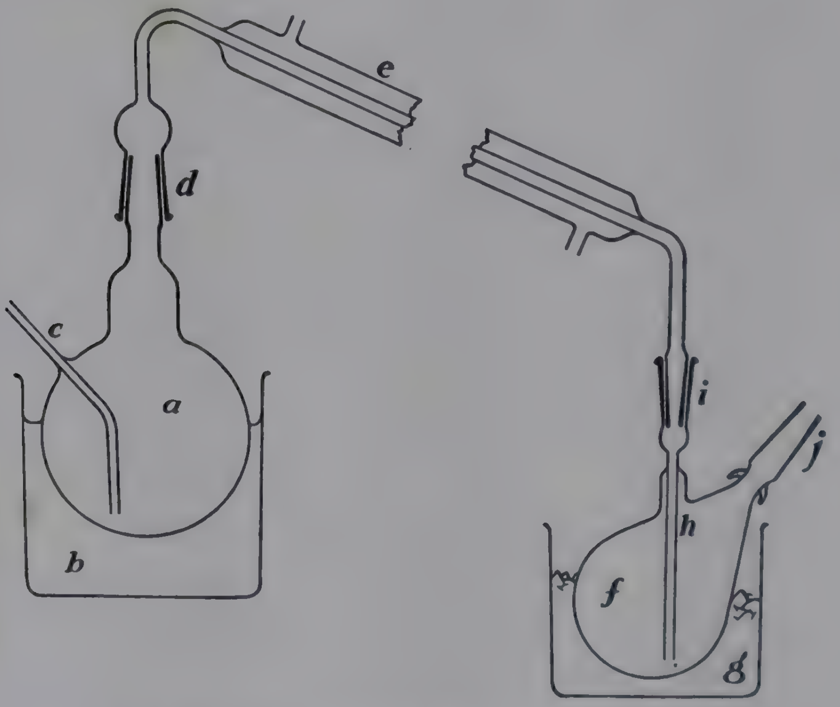

Fig. 692.—Apparatus for steam distillation.

Apparatus for Steam Distillation (Fig. 692).—The apparatus consists of: *a*, a distilling flask with a capacity of 2 liters; *b*, a bath for boiling water; *c*, a glass tube sealed into the side of the distilling flask for the delivery of the steam; *d*, an interchangeable No. 15 ground glass joint for connecting the distilling flask with the condenser; *e*, a water-cooled condenser; *f*, the bulb of the rectification flask, for the reception of the distillate from the tissues; *g*, an ice bath; *h*, a glass tube sealed into the side of the rectification flask; *i*, an interchangeable No. 7 ground glass joint for connecting the condenser with the bulb of the rectification flask which is used as a receiver for the distillate; and *j*, an interchangeable No. 9 ground glass joint used during rectification (Fig. 693).

Technic of Distillation of Volatile Organic Liquids From Tissues.—

Place the tissues, removed at autopsy, in glass jars, seal tightly, and place in a refrigerator.

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[5] Arch. Path. 17: 510, 1934.

When ice cold, quickly grind 600 gm. in a grinder previously cooled by the application of ice.

Rapidly weigh 500 gm. of the ground material.

Mix with 200 c.c. of ice-cold water.

Introduce into the 2-liter distilling flask (Fig. 692*a*).

Add an additional 300 c.c. of water and 1 c.c. of liquid petrolatum.

Set up the apparatus for steam distillation as indicated in Fig. 692.

Continue the steam distillation until about 200 c.c. of distillate have collected in the ice-cooled bulb of the rectification flask (Fig. 692). Experiments have shown that this amount is ample for recovery of practically all the volatile organic liquid that may be present in the tissues.

Remove the rectification bulb and its contents from the distilling chain, and keep cold by surrounding with cracked ice.

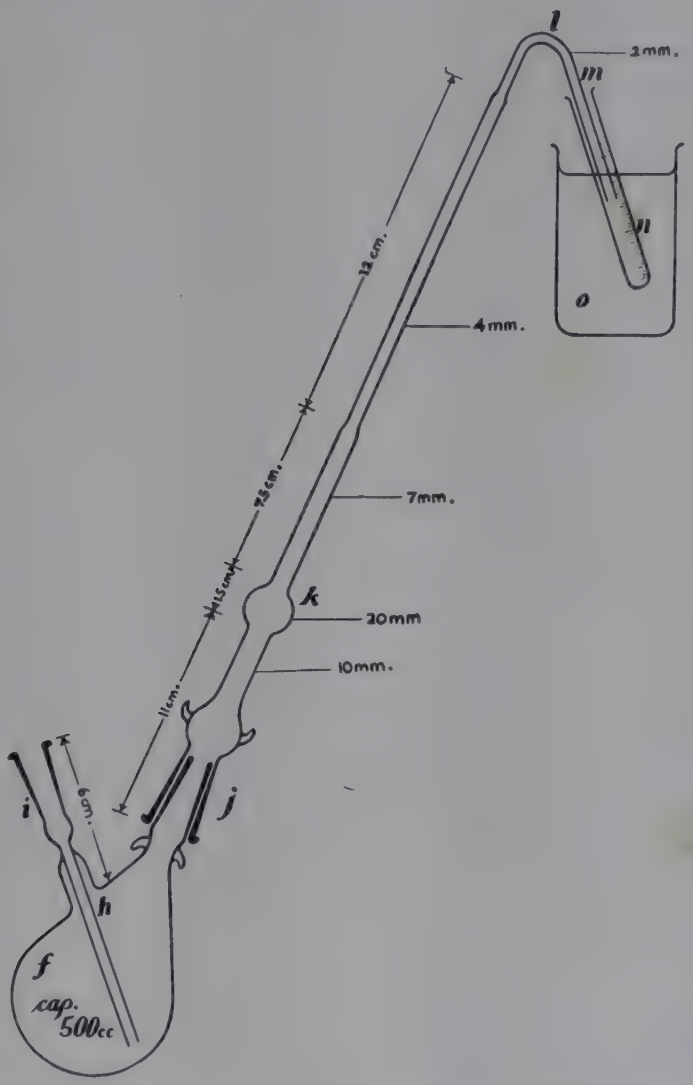

Fig. 693.—Rectification flask for isolation of volatile organic fluids.

Gradually add to the distillate in the flask 250 gm. of anhydrous calcium chloride, keeping the solution well mixed to prevent warming. The purpose of the addition of the calcium chloride is to effect a salting out of the volatile liquid present and also to lower the vapor pressure of the water.

Add about 1 gm. of granulated zinc to prevent bumping.

Set up the rectification bulb and its contents for rectification as indicated in Fig. 693.

Rectification Flask for Isolation of Volatile Organic Liquids (Fig. 693).

The apparatus consists of: *f*, a distillation bulb (capacity, 500 c.c.); *h*, a glass tube fused into the side of the distillation bulb, which acts as a safety-valve; *j*, an interchangeable No. 9 ground glass joint; *k-l*, a reflux condenser (air-cooled); *m*, a microcondenser (cooled by a freezing bath); *n*, a tube for collection of the distillate, the inside diameter of which measures 6 mm. and which is graduated in twentieths of a c.c.; and *o*, a solid carbon dioxide-acetone cooling bath.

The rectification flask used in these determinations is slightly modified from that used by Gettler and Siegel.[1] It is constructed in two parts, *f* and *klm*, connected by a ground glass joint (*j*). This change in structure facilitates the introduction and removal of material into and from the flask.

Technic of Isolation of Volatile Organic Liquids From Tissue Distillate.—

Rectification.—In order to obtain the best results it is essential that the condenser parts *k*, *l*, and *m* of the rectification flask be perfectly clean. Treat with hot concentrated nitric acid, or let them stand overnight in a cleaning mixture of sulphuric and chromic acids. Before attaching the condenser to the bulb of the rectification flask, thoroughly dry the inner surface of the portion of *k*, *l*, and *m* by playing a gentle flame over the outer surface. Keep the receiving tube (*n*) in a cooling bath (*o*). With volatile liquids of extremely low freezing points, the cooling bath consists of carbon dioxide snow in acetone. For the liquids which freeze at a point only a little below 0° C. an ice and salt mixture is used. For benzene which freezes at 5.4° C. ice-water is satisfactory.

To start the rectification, heat the material in the bulb of the flask carefully and slowly, using an asbestos-centered wire gauze and a microburner. As soon as the liquid in the bulb begins to boil, regulate the flame so that the liquid boils gently for about 15 minutes. During this time, the steam (as indicated by a visible rise of the condensate or by running the hand along the tube of the flask and determining the height of steam by the temperature of the tube) must not be permitted to rise beyond *k*. This is done by properly regulating the flame. The part of the tube from *k* to *l* acts as a reflux for the steam, whereas the vapors of the volatile liquid which rise past *l* condense and collect in the receiver *n*.

Continue gently boiling for 15 minutes.

Gradually increase the flame so that the steam is made to rise slowly (in one to two minutes) until it just passes the bend of tube (*l*).

From this point the procedure varies according to the cooling bath used. If a carbon dioxide snow-acetone mixture is used, continue the distillation at the same constant rate. After the volatile organic liquid has distilled over, steam will follow. Beyond *l* the steam will condense and freeze. Ice will clog the tube (*m*). The pressure produced within the flask will force the liquid in the bulb out through the safety-tube (*h*). This occurrence indicates the end-point in the distillation.

If an ice and salt or a plain ice-water cooling bath is used, the water vapor rising into *m* may not freeze and clog the tube, and hence no back pressure will be produced. In this case the end-point of the distillation is signified when the bend (*l*) attains the temperature of boiling water. Rectification is now completed. The volatile liquid, if present, will be found in the calibrated receiver (*n*).

Remove the receiving tube from the cooling bath.

Stopper lightly and allow to stand at room temperature until the ice present has melted.

At once centrifugate the tube and contents for 2 minutes.

Place in ice-cold water for 5 minutes.

Read the volume of the layer of isolated liquid

Identification of the Isolated Liquid.—

The following tests may be applied to identify the liquid:

(a) microdetermination of the boiling point by the method of Emich;[2] also described by Gettler, Niederl, and Benedetti-Pichler;[3]

(b) microdetermination of the molecular weight;[4]

(c) microanalytic determination of carbon and hydrogen;[5]

(d) microdetermination of halogen.[6]

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[1] Arch. Path. 17: 510, 1934.

[2] Monatsh. f. Chem. 38: 219, 1917.

[3] Mikrochemie 11: 173, 1932; J. Am. Chem. Soc. 54: 1479, 1932.

[4] Niederl, J. B.: Ztschr. f. anal.: Chem. 77: 169, 1929.

[5] Niederl, J. B., and Roth, R. T.: Indust. & Engin. Chem. 6: 272, 1934.

[6] Elek, A., and Dill, D. W.: J. Am. Chem. Soc. 55: 2552, 1933.

Gettler and Siegel used the microdetermination of the boiling point for the identification of the isolated liquid. They are convinced, through the evidence of experiments, that the isolated volatile liquid need not be dried for use in the determination of the boiling point.

They succeeded in isolating the following from tissue: ethyl chloride, ethylene chloride, propylene dichloride, carbon tetrachloride, dichlor-difluormethane, chloroform, carbon bisulphide, benzene, and diethyl ether. Identification of the particular liquid present was accomplished by microdeterminations of the boiling point. One set of values obtained is charted in Table 220.

TABLE 220

RECOVERY AND BOILING POINT OF ISOLATED VOLATILE ORGANIC LIQUIDS

VOLATILE LIQUID	BOILING POINT OF THE LIQUID, ° C.	AMOUNT OF LIQUID ADDED TO 200 C.C. OF WATER, C.C.	AMOUNT OF LIQUID ADDED TO 500 GM. OF TISSUE, C.C.	AMOUNT OF LIQUID RECOVERED, C.C.	BOILING POINT OF THE ISOLATED LIQUID, ° C.
Ethyl chloride	12.2	0.3	--	0.08	12.2
	12.2	--	0.3	0.05	12.4
Ethylene chloride	83.2	0.3	--	0.27	83.3
	83.2	--	0.3	0.22	83.1
Propylene dichloride	95.6	0.3	--	0.27	95.7
	95.6	--	0.3	0.19	95.7
Carbon tetrachloride	76.7	0.3	--	0.27	76.1
	76.7	--	0.3	0.05	68.0*
	76.7	--	1.0	0.10	74.0
Dichlor-difluormethane	39.4	0.3	--	0.26	39.4
	39.4	--	0.3	0.17	40.0
Chloroform	62.1	0.3	--	0.21	62.1
	62.1	--	0.3	0.09	62.2
Carbon bisulphide	46.1	0.3	--	0.27	46.0
	46.1	--	0.3	0.09	46.0
Benzene	80.5	0.3	--	0.25	80.1
	80.5	--	0.3	0.10	80.2
Diethyl ether	34.8	0.3	--	0.25	34.9
	34.8	--	0.3	0.24	34.7

*Carbon tetrachloride isolated from tissues always gave an abnormally low boiling point, which proved to be due to the fact that some of the tetrachloride is converted into chloroform, the mixture of chloroform and carbon tetrachloride giving the abnormally low boiling point. By means of Emich's microfractionating tube[1] the chloroform and the carbon tetrachloride can be separated. If this procedure is carried out, the fraction containing the tetrachloride gives the correct boiling point.

ISOLATION OF ETHER FROM HUMAN TISSUES

The work of Gettler and Siegel[2] has facilitated the isolation of ethyl ether from human tissues. Up to the time of their publication in 1934, methods which were based on oxidation-reduction reactions were used for the determination of ether. But these were unsatisfactory because other organic substances react just as ether does toward these oxidizing agents. The reliance of the pathologist or toxicologist on his sense of smell, when the body cavities are opened, is apt to be quite misleading. Gettler, Niederl, and Benedetti-Pichler[3] devised a specially constructed rectification flask to isolate ether from tissue distillates. Changes have been made in this microrectification flask as follows:

1. The bulb of the flask is of larger capacity (300 c.c.) which enables one to use the entire original tissue distillate rather than small aliquot portions. This enables one to measure the volume of ether isolated.

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[1] Gettler, A. O., and Fine, J.: Indust. & Engin. Chem. **11**: 469, 1939.

[2] Arch. Path. **17**: 510, 1934.

[3] Mikrochemie **11**: 173, 1932; J. Am. Chem. Soc. **54**: 1479, 1932.

2. A glass tube running to the bottom of the flask is fused into the side of the flask, which facilitates the introduction of the material from which the ether is to be isolated and serves as a safety tube during the distillation and also makes it easier to empty and clean the flask.

3. The uppermost S-shaped part is straightened out and points downward at an angle of 45 degrees to the main tube of the flask. This was done so that all the ether could be collected in one portion rather than in a series of successive drops. See Fig. 694.

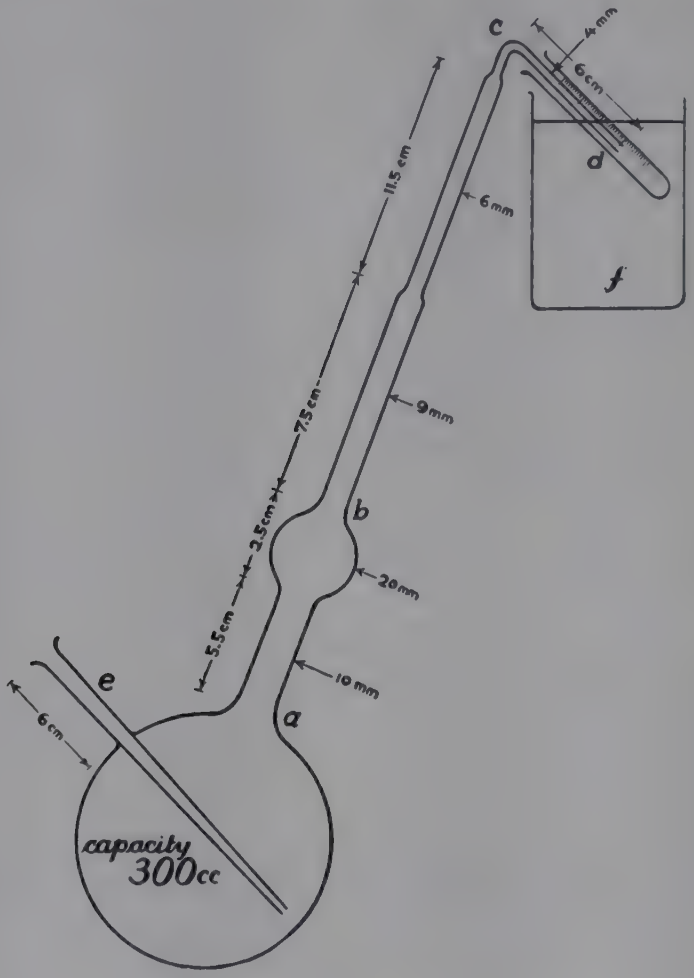

Fig. 694.—Microrectification flask.

Method of Gettler and Siegel[1]

Tissues and organs removed at autopsy are placed quickly in glass jars, tightly sealed and put in a refrigerator. When ice cold, quickly grind up about 600 gm. in a grinder previously cooled by applying ice. Of this ground material, rapidly weigh 500 gm., mix with 200 c.c. of ice cold water, and place in a 2-liter flask.

Add an additional 300 c.c. of water and 1 c.c. of liquid petrolatum. Distill the mixture with steam, using a long, well cooled condenser, the tip of which has been bent to resemble an adapter. The bent tip should dip into 25 c.c. of ice water contained in the receiving flask, the latter being at all times entirely surrounded by ice. Collect 200 c.c. of distillate. This is ample for recovering all the ether that is present in the tissues. The distillate is now ready for the isolation of the ether.

Use the rectification flask already described. It must be clean and part of it (from *b* to *d*) must be dried by playing a gentle flame over the outside surface.

Pour the entire 200 c.c. of distillate through the safety tube (*e*) into the rectification flask. Add 1 gm. of granulated zinc. The receiving tube (*d*), which is 6 mm. in diameter on the inside and is graduated in twentieths of a cubic centimeter, is surrounded by a bath

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[1] Arch. Path. 17: 510, 1934.

(*f*) of solid carbon dioxide in acetone, and should contain about 0.15 c.c. of water (not more), so that the ether is collected over water as a separate layer. Now heat the rectification flask carefully and slowly, using an asbestos centered wire gauze and a microburner. As soon as the solution begins to boil, regulate the flame so that the liquid boils gently in the bulb for about fifteen minutes. During this time the steam (as indicated by a visible rise of the condensate or by running the hand along the tube and determining the height of the steam by the temperature) must not be permitted to rise beyond *b*. This is done by properly regulating the flame. The part of the tube from *a* to *c* acts as a reflux for the steam, while the ether vapors rise past *c*, condense and collect in the receiver. After mild boiling has been continued for fifteen minutes, gradually increase the flame so that the steam is made to rise slowly (in one minute) until it just passes the bend *c*. Rectification is now completed. The ether, if present, will be found unfrozen in the calibrated receiver. It sometimes happens that water vapor freezes in the tube beyond *c*, clogging it up; back pressure developed by continued heating will force the distillate out through the safety tube *c*. In that case, remove the flame; then remove the receiving tube, and warm the portion of the tube from *c* to *d* with the fingers. When the ice melts, it will be sucked back into the flask. Replace the receiving tube and continue the rectification.

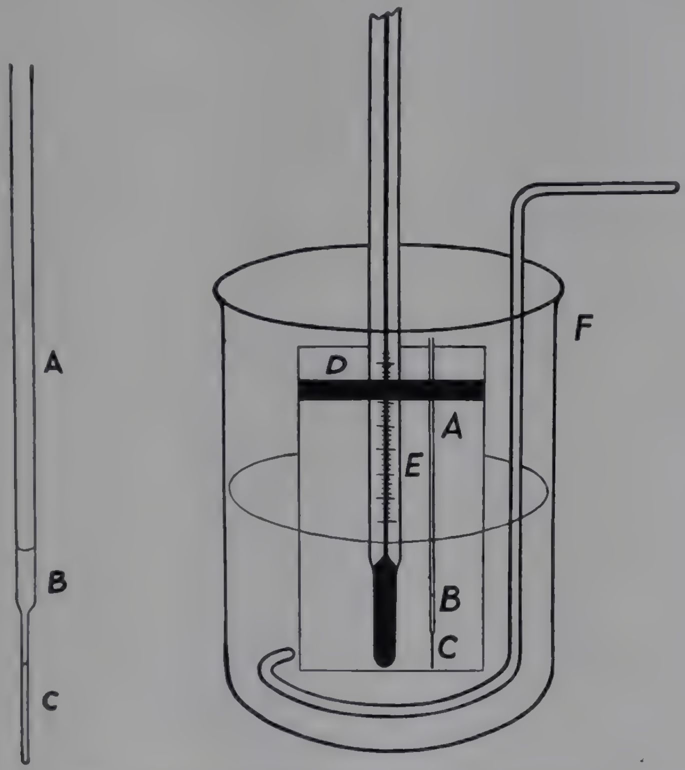

Fig. 695.—Microboiling point apparatus.

When the rectification is completed, as indicated by steam having passed the bend (c), remove the receiving tube from the acetone bath, lightly stopper, and allow it to stand at room temperature until any ice in the calibrated receiving tube melts. Read the volume of recovered ether (upper layer) immediately.

In order to identify this upper layer as ether, determine the micro-boiling point. Gettler and Siegel were convinced that the isolated ether need not be dried for determination of the boiling point. The isolated ether can be sealed in a capillary tube and kept as corpus delicti.

Determination of Boiling Point.[2]—Draw out a capillary tube (Fig. 695*A*), from 1.5 to 2 mm. in diameter, into a finer capillary tube at one end (*C*), about 8 mm. in length. Introduce the end (*C*) of the capillary tube into the ether to be tested. Ether is drawn into the tube by capillary action. Care must be taken to allow only a small droplet to enter. Then seal the tube by bringing the tip of it into the edge of a Bunsen flame for a second. As the walls of the tube are extremely thin, they fuse together almost instantly. Sealing in this way always leaves a bubble of air in the tip of the tube, between the sealed portion of glass and the drop of liquid (Fig. 695*C*). This bubble of gas serves to prevent superheating of the liquid during the determination of the boiling point. Mount the capillary tube (A) containing the drop of liquid and a thermometer (E) on a microscope slide and set up in a beaker (F) containing water, together with a stirrer. Raise the temperature of the bath slowly and uniformly. According to Emich, the true boiling point of a pure liquid is the temperature at which the droplet in the capillary reaches the surface of the bath. This is true only, however, if the bubble of gas in the tip of the tube is so small that the effect of its expansion is negligible, and also if the temperature of the bath is raised regularly and uniformly throughout.

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[2] Emich, F.: Monatsh. f. Chem. 38: 219, 1917.

A series of determinations was made on the boiling point of pure, dry ether as well as of ether that had been in contact with water for an indefinite period. The boiling points obtained were: pure, dry ether, from 34.8° to 35° C., and moist ether, from 34.7° to 35° C. Since the boiling point of moist ether is practically the same as that of dry ether, it is not necessary to dry a sample of ether before determining its boiling point.

Explaining, Gettler and Siegel showed in Table 221 how much ether was recovered after known quantities were added to 500 gram portions of brain tissue and liver tissue.

This micromethod enabled these workers to isolate as small a quantity as 0.03 c.c. of ether when 0.06 c.c. was added to 500 gm. of tissue. The determinations of the microboiling point served to identify the isolated liquid as ethyl ether.

TABLE 221.—STUDY OF RECOVERY OF ETHER

NO.	TISSUE USED 500 GM.	ETHER ADDED, C.C.	ETHER RECOVERED, C.C.	ETHER LOST, C.C.	% RECOVERY
1	Brain	0.06	0.03	0.03	50
2	Brain	0.06	0.03	0.03	50
3	Brain	0.12	0.05	0.07	42
4	Brain	0.12	0.07	0.05	60
5	Brain	0.15	0.10	0.05	67
6	Brain	0.15	0.09	0.06	60
7	Liver	0.30	0.24	0.06	80
8	Brain	0.30	0.25	0.05	83
9	Brain	0.45	0.37	0.08	82
10	Brain	0.45	0.38	0.07	84
11	Liver	0.60	0.47	0.13	78
12	Brain	0.72	0.63	0.09	88
13	Brain	0.75	0.67	0.08	89
14	Brain	0.90	0.78	0.12	87
15	Brain	0.90	0.74	0.16	82

Distillation Capillary Method Useful to Determine Boiling Points

A further contribution to the isolation of volatile fluids was made by Gettler and Fine.[3] They showed that mixtures of low boiling liquids, in volumes as small as 0.02 to 0.1 c.c. can be fractionally distilled by means of the apparatus described below.

Preparation of Distillation Capillary (Fig. 696).—Heat a piece of Pyrex glass tubing, A, about 8 mm. in outside diameter, and with 1 mm. wall, in the blast lamp and draw out to a capillary, B, somewhat longer than 10 cm., with a uniform inner bore of approximately 2 mm. Cut off a 10 cm. section, C, of this capillary. About 5 cm. from one end, draw out the capillary to a much finer capillary, D, 0.25 to 0.5 mm. in outside diameter, and about 7 cm. long. Cut off any excess length. Seal the end of the finer capillary by heating in the flame.

Then seal the end of the larger capillary, using a small blast flame, and keep the glass soft by holding it in the flame until a hollow bulb about 5 to 6 mm. in diameter (0.06 to 0.1 c.c. capacity) forms, E, owing to the increased pressure of the heated air within. The size of the bulb will depend upon the length of time that it is kept in the flame. Excessive heating must be avoided, since this will gradually enlarge the bulb to the point of bursting. When cool, break off the tip of the fine capillary. Soften the stem of the fine capillary at a point 1 to 2 cm. from the larger capillary by heating in a luminous flame, and bend to an angle of about 45°. The distillation capillary is now ready for use.

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[3] Indust. & Engin. Chem. 11: 469, 1939.

Filling the Distillation Capillary (Fig. 697).—Introduce the drop of liquid to be fractionated into the distillation capillary in the following manner:

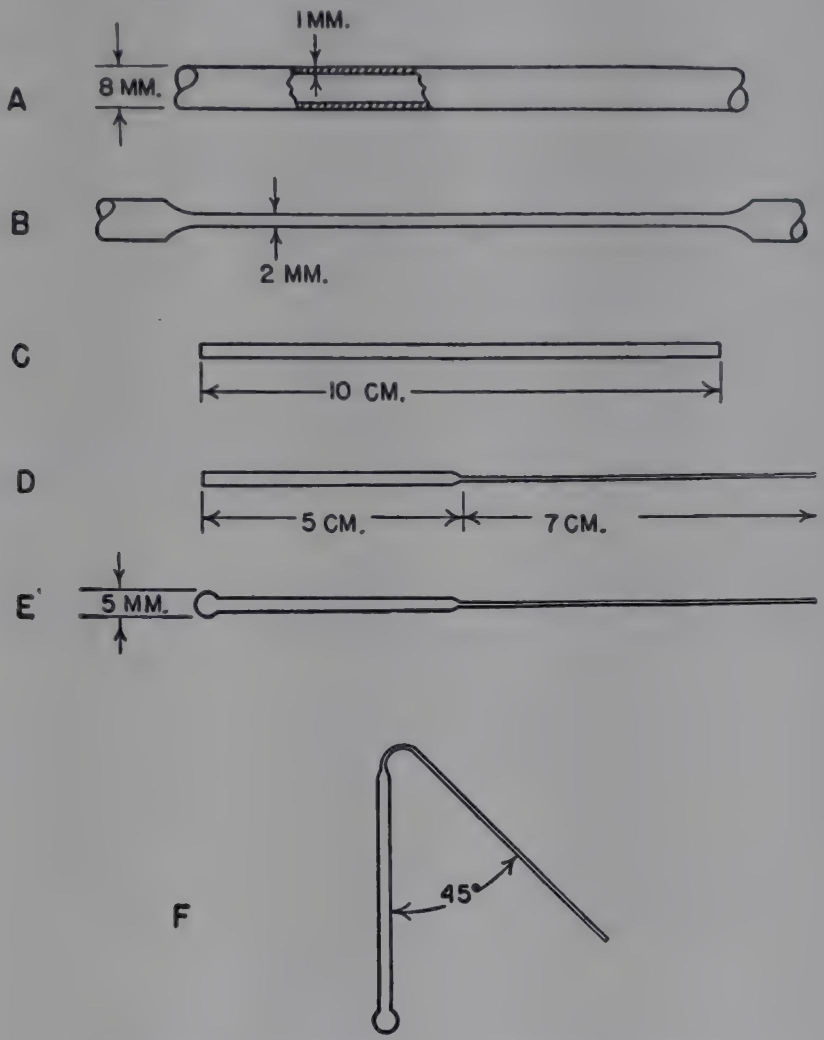

Fig. 696.—Preparation of distillation capillary.

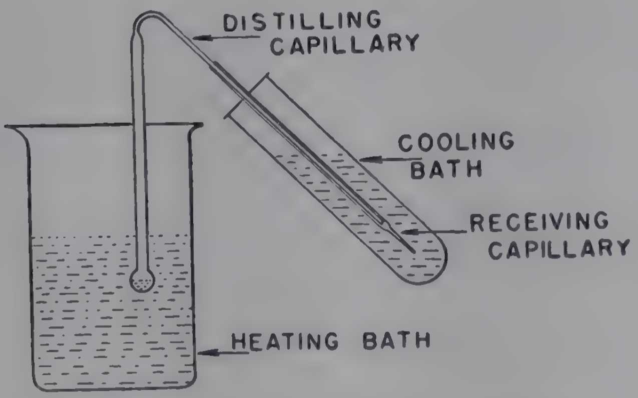

Fig. 697.—Filling the distillation capillary.

Warm the bulb of the distillation capillary by immersing for a few minutes in boiling water. While the bulb is still warm, dip the fine capillary tip into the drop of liquid to be fractionated, and then apply a cooling bath (solid carbon dioxide and acetone) to the bulb. The decreased air pressure within, due to the cooling, causes the liquid to flow up the fine capillary, down the larger capillary, and into the bulb of the apparatus. Should any of the liquid remain in the stem, it is easily forced into the bulb by shaking the apparatus two or three times, as is customary with clinical thermometers. For a successful distillation the bulb should not be more than half full of liquid. In order to remove all traces of adhering liquid from the fine capillary arm, carefully warm the arm, while keeping the bulb containing the bulk of the liquid in the cooling mixture.

The Distillation.—Use a test tube, 6 cm. long, partly filled with ice water, to cool the distillate-receiving capillary. For very low boiling liquids (30° C. or less) use a small Dewar flask containing liquid air.

Introduce a capillary, 9 cm. long, having an inside diameter just a trifle larger than the outside diameter of the fine arm of the distillation capillary, into the cooling bath, with its sealed end resting on the bottom of the test tube. Introduce the fine stem of the distillation capillary into the receiving capillary as far as it will go. The entire setup is held by means of the water-containing test tube during the distillation. Now insert the bulb of the distillation capillary into cold water contained in a small beaker. Then gradually and very slowly heat the water until a ring of condensate is seen rising up the column and condensing in the receiving capillary. At this point the flame should be at once removed, in order to keep the temperature of the water from rising too high. The distillation meanwhile is allowed to continue until a layer not more than 2 mm. thick (0.01 c.c. volume) has collected in the receiving capillary. At this point quickly withdraw the distillation capillary from the receiving capillary by means of a glass rod supporting the curved part of the distillation capillary, and quickly place the bulb in the solid carbon dioxide-acetone cooling mixture, in order to suck back into the bulb any liquid remaining in the capillary portion of the apparatus.

Store the receiving capillary containing this first fraction in the cooling mixture until ready for identification tests, such as the boiling point determination. Then continue fractional distillation of the remaining liquid in the bulb as described above. Not more than 2 mm. of each fraction should be collected in the receiving capillaries. Five to six fractions can be obtained in this way from 0.06 c.c. of liquid.

With this method, Table 222 shows experimental results in boiling points of different volatile substances.

TABLE 222.—EXPERIMENTAL RESULTS

C.C.	MIXTURES OF LIQUIDS USED	BOILING POINTS OF PURE LIQUIDS ° C.	EXPT.	BOILING POINTS OF SUCCESSIVE FRACTIONS ° C.
0.02	Ethyl ether	34.5	1	34.5, 35.0, 49.5, 55.8
0.02	Acetone	56.5	2	34.5, 35.3, 43.0, 55.5, 56.0
0.03	Methyl alcohol	64.6	1	33.0, 35.5, 37.0, 41.5, 60.5, 63.5
0.03	Methyl formate	31.5	2	32.0, 33.5, 38.5, 42.5, 63.2, 64.0
0.03	Ethyl ether	34.5	1	21.5, 21.5, 22.0, 33.0, 34.0
0.03	Acetaldehyde	21.5	2	21.0, 21.0, 22.5, 33.5, 34.0
0.03	*n*-Propyl chloride	46.4	1	37.5, 37.5, 42.5, 43.5, 45.0
0.03	Iso-Propyl chloride	36.5	2	36.0, 37.0, 40.5, 44.0, 46.5, 46.8

This apparatus can be used not only for the isolation from tissues of ether, but also for all other easily volatile liquids; that is, liquids with boiling points below that of water, such as carbon tetrachloride, propylene chloride, ethylene chloride, ethyl chloride, and many others.*

DETECTION OF PHENOLS

Distill tissue by steam distillation in the usual manner (page 2243). Test the distillate for phenol by the following tests.

1. **Ferric Chloride Test.**—

To 10 c.c. of the distillate, add a few drops of 10% ferric chloride. Phenol gives a blue-violet color. Cresols give a green to blue color.

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*This apparatus is supplied by Eck & Krebs, 1313 West 24th St., New York City. It is listed as Rectification Apparatus, Micro, Gettler and Siegel, No. 3540.

2. Bromine Test.—

To 10 c.c. of the distillate, add 2 to 3 c.c. of saturated bromine water. Brominated phenol will precipitate (yellow) 2,4,6 tribromphenol. Melting point is 94° C.

3. Millon's Test.—

Millon's Reagent.—

Dissolve 10 grams mercury in 20 grams nitric acid, c. p.
Dilute with an equal volume of distilled water.
Allow to stand twenty-four hours.
Decant.

To 10 c.c. of distillate add 1 c.c. of Millon's reagent.

If no color develops apply gentle heat. A red color or precipitate may indicate phenols, cresols, or salicylates.

Since phenolic compounds may be produced by putrefaction and thus give a positive Millon reaction, caution must be exerted in examining putrified cadavers for phenol.

GROUP II. ACID-ETHER EXTRACTION

Carry out steam distillation as described on page 2243. Use the aqueous residue left in the flask after steam distillation to extract the nonvolatile poisons.

Place the residue in a large evaporating dish and add 2 to 3 times its volume of 95% ethyl alcohol.

Place on a steam bath for several hours. The alcohol dissolves most poisons. Heat and alcohol combined tend to denature the proteins present in the specimen, rendering them insoluble.

Filter. Heat the filtrate to volatilize all the alcohol.

Leach the syrupy residue with hot distilled water many times and then extract with several portions of ether. The ether extracts and isolates acid-ether soluble poisons. The tartaric acid, used in the original distillation process, is still a sufficient acidifying agent. It is well, however, to check the solution with litmus paper, to see if it is acid.

Collect these several ether extracts and evaporate on a steam bath.

Test the residue for the various common poisons in this group as described below.

In most cases, the residue is still impure; it has a yellow tinge due to nitrogenous impurities. This impure residue may be purified with selective solvents, or, better, by sublimation which usually yields fine pure crystalline material.

DETECTION OF BARBITURATES IN TISSUE AND URINE

Gettler Method

Gettler[1] gives the following procedure for isolation of barbiturates.

Isolation from Urine and Tissues.—The urine is best adapted for a rapid qualitative analysis for barbiturates. Extract 100 c.c. of urine (HCl acidified) in a separatory funnel with 100 c.c. of ether. Remove the ether layer containing the barbiturate and wash with two successive 10 c.c. portions of water. Pour the washed ether layer into a beaker, and place on a boiling water bath, until all the ether has evaporated. A white, and usually crystalline, residue indicates the presence of barbiturates. In the analysis of tissues, the procedure is somewhat more complicated, in that an alcohol extraction precedes the ether extraction, as described in the classical Stas-Otto procedure.

Identification of the Residue.—(a) Dissolve a small quantity of the purified residue in about five drops of chloroform and place in a micro test tube. Add a drop of 1 per cent cobalt acetate in methyl alcohol, and mix the contents. Then stratify two or three drops of 5 per cent isopropyl amine in methyl alcohol over the mixture. A blue-violet color develops between the stratified layers if barbiturates are present.

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[1] Am. J. Clin. Path. 13: 169, 1943.

Most barbiturates are extracted in crystalline form from acid-ether. Since seconal usually does not appear in crystalline form, but has an oily appearance, it is imperative that the cobalt and Millon tests be made even though there are no visible crystals in the residue. The presence of color matter and impurities inhibits the blue-violet phase of the cobalt test. If, therefore, the cobalt test is negative, it is well to purify further by sublimation, or by hot and cold water or solvent recrystallization, or by shaking out with activated charcoal and filtering, and then to repeat the test. If the test is again negative, it is now at least a more reliable indication of absence of barbiturates.

(b) Place a small quantity of the residue in a test tube, and dissolve in a little hot water. Then add a drop of Millon's reagent. A voluminous white precipitate, which dissolves on adding an excess of the reagent, indicates barbiturates.

(c) In order to establish which one of the barbiturates is present, the melting point of the extracted material is determined. Since the residue very often is not pure enough for a melting point determination, it must first be sublimated. Place a glass slide with a crystal of the sublimate on the stage of a microscope, which is heated electrically, and is supplied with a thermometer. Observe the melting point of the crystal in a normal manner, through the microscope.

Once an identification is made, confirm by performing a mixed melting point determination with another crystal supposedly identical with the unknown. Obtain a derivative with either xanthydrol or *p*-nitro benzoyl chloride, and compare melting points with known compounds.

SUBSTANCE	MELTING POINT	MELTING POINT OF *p*-NITRO BENZOL DERIVATIVE
Veronal	190	194
Luminal	175	184
Evipal	144	115
Amytal	154	146
Dial	170	193

Quantitative Determination.—(a) By weighing the extracted, purified (by repeated extractions), and dried barbiturate.

(b) By determining the nitrogen content of the purified extract, from which the quantity of barbiturate may be calculated.

(c) Colorimetrically, using the color produced by cobalt acetate in methyl alcohol when added to a chloroform solution of the barbiturate, and made alkaline by a methyl alcohol solution of isopropyl amine. The extracted material must also be purified before this test is applied.

All three methods are not strictly quantitative, because there is an unavoidable loss during purification.

Distribution of the Barbiturates in the Various Tissues.—Quantitative chemical analyses revealed that the barbiturates are more or less evenly distributed in the blood, brain, liver, kidneys and muscles. A difference of more than 2 mg. in 100 gm. of any of the tissues is a rare occurrence.

The Minimum Lethal Tissue and Blood Level.—Over 400 barbiturate deaths have been analyzed in the chemical laboratories of the Chief Medical Examiner's Office of New York City over a ten-year period. The data show that the barbiturates can be divided into four groups, from the standpoint of the minimum lethal tissue or blood level. In 100 gm. of tissue or blood we find 1.5 mg. as the minimum fatal concentration for Amytal and Evipal; 4.0 mg. for Nembutal, Neonal, Ortal, Phanodorn and Seconal; 8.0 mg. for Dial, Ipral, Luminal, Numal (Allonal) and Propanol; 18.0 mg. for Veronal. The low tissue content of Amytal and Evipal is due to their greater toxicity, and to their more rapid destruction in the living body. The elimination from the system during life is quite rapid (thirty-six hours) for barbiturates having a low lethal tissue concentration such as Amytal and Evipal, and slow (four to five days) for barbiturates, such as Veronal, having a higher lethal tissue concentration.

BARBITURATES

Spectrophotometric Method of Goldbaum[1]

Both qualitative and quantitative tests for barbiturates can be made by a study of their characteristic ultraviolet absorption spectra. Concentrations as low as 1 mg. in 100 gm. of blood or tissue can be determined with an accuracy of approximately 95 per cent.

Principle.—

Absorption curves are determined in a Beckman universal quartz spectrophotometer using 1 cm. cells. Measurements are reported as density (2 - log G).

Fig. 698 shows the absorption spectra of some representative barbiturates. An intense maximum is present at wave length 255 for those barbiturates having a malonyl urea ring. At wave length 255 mμ there is a straight line relationship between concentration and density (2 - log G).

Reagents.—

Redistilled Chloroform.—

Acetate Buffer pH 5.0.—

Mix 59 c.c. of 0.1 N acetic acid
with 141 c.c. of 0.1 N sodium acetate.

0.5 N Sodium Hydroxide Solution.—

Reference Solution.—

0.5 N sodium hydroxide solution saturated with chloroform (or with ether for brain analysis).

Ether.—

Technic.—

(I) **In Plasma and Whole Blood.—**

Using a buret admit 15 c.c. of redistilled chloroform to a 50 c.c. separatory funnel.

Add 2 c.c. of acetate buffer pH 5.0.

Add 1 c.c. of blood or plasma.

Shake for 1 minute.

Allow the layers to separate. If an emulsion forms, which is rare, it can be broken by centrifugalizing the specimen.

Filter the chloroform through Whatman No. 42 filter paper into a calibrated 15 c.c. centrifuge tube.

Collect 10 c.c. of clear filtrate.

Transfer the chloroform to another clean 50 c.c. separatory funnel.

Add 5 c.c. of 0.5 N sodium hydroxide solution and shake the separatory funnel for about 1 minute.

Allow the layers to separate.

Discard the chloroform layer.

Transfer the alkaline layer to a 10 c.c. Pyrex tube.

Centrifuge for 2 to 3 minutes to bring down the suspended droplets of chloroform. The solution should now be crystal clear.

Pipette off 4 c.c. of this clear alkaline solution and use 1 c.c. to rinse the Beckman cuvette, the remaining 3 c.c. to fill the cuvette.

Compare the unknown alkaline solution in the Beckman instrument with the reference solution.

In using the Beckman spectrophotometer, put the alkaline solution containing the barbiturates in one of the cells and the reagent blank in the other. Adjust the instrument to 100 per cent transmission with the reagent blank. Then put the unknown in the instru-

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[1] Goldbaum, L. R., Army Medical School. Personal communication.

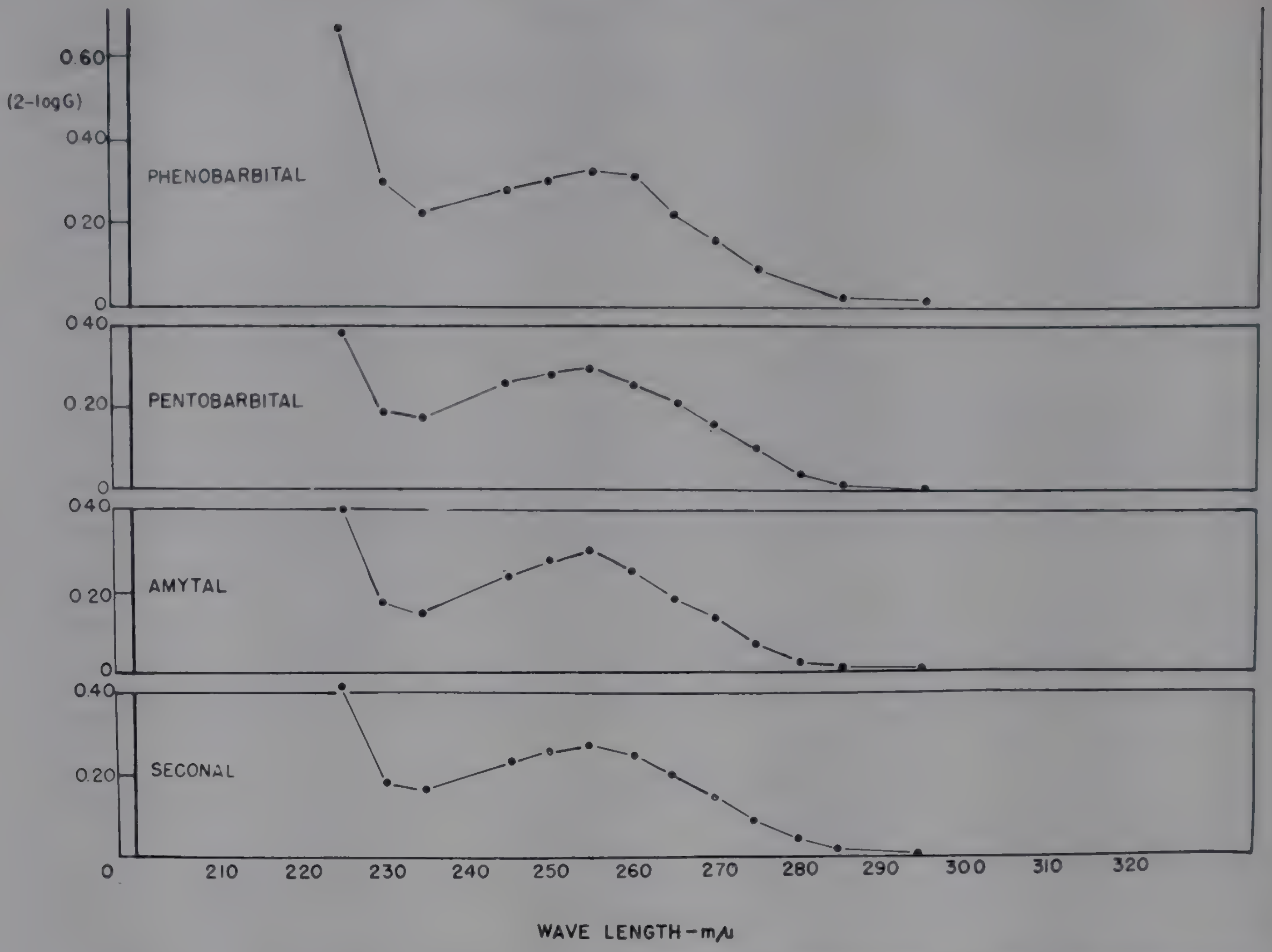

Fig. 698.—Absorption spectra of some representative barbiturates in 0.5 N sodium hydroxide solution. Each contains a malonyl urea ring structure and is characterized by an intense ultraviolet absorption spectrum with a maximum at 255 mμ and a minimum at 235 mμ. (Courtesy Dr. L. R. Goldbaum.)

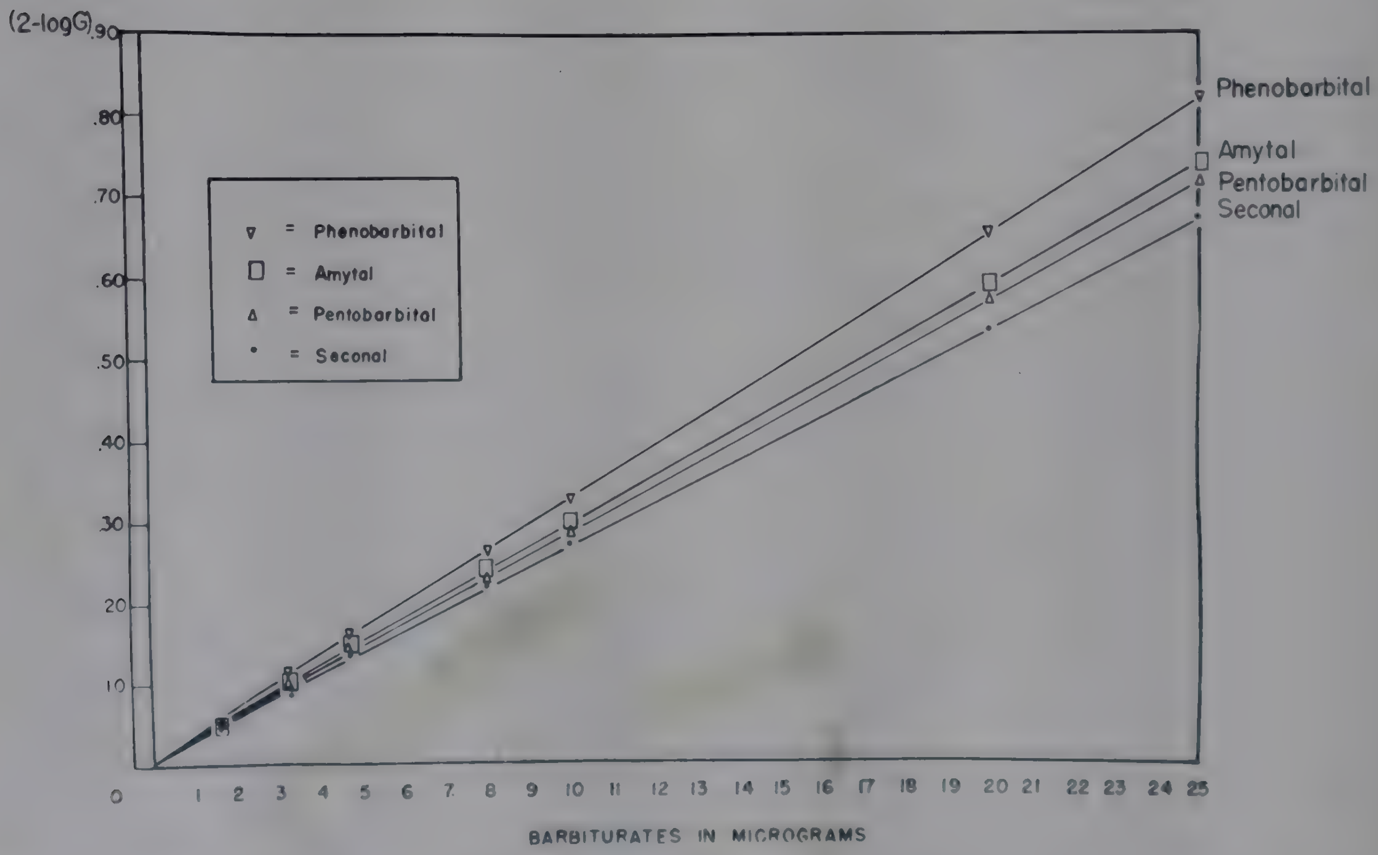

Fig. 699.—Barbiturates in micrograms obtained by the Goldbaum method. At maximal absorption, 255 mμ, the Beer-Lambert law relating to concentration and optical density is shown to be linear up to at least 20 micrograms per c.c. (Courtesy Dr. L. R. Goldbaum.)

ment and determine its transmission. Repeat at all wave lengths so as to obtain the entire absorption spectrum as shown in Fig. 698.

The maximum absorption of barbiturates is at 255 mμ; this means that they have their greatest density value at this wave length, and therefore the greatest sensitivity.

To identify the barbiturates, determine the whole band to see if it has a maximum at 255 mμ; then obtain its optical density at 255 mμ. Find the quantity by comparing either with a standard solution or with a known graph as in Fig. 699.

(II) **In Liver, Kidney, and Muscle.—**

Render 5 gm. of tissue homogeneous in a tissue blender using acetate buffer of pH 5.0, then make up the volume to the mark 25 c.c. in a volumetric flask, using the acetate buffer.

Pipette a 5 c.c. aliquot, equivalent to 1 gm., into a separatory funnel containing 15 c.c. of chloroform.

Carry out the procedure as in (I) above.

(III) **In Brain.—**

Brain cannot be extracted with chloroform because interfering substances produce emulsions and cause a high blank. Use ether instead of chloroform for the extraction of barbiturates from brain.

Render 5 gm. of brain tissue homogeneous using acetate buffer of pH 5.0.

Pipette 10 c.c. of this mixture, equivalent to 2 gm. of brain, into a 125 c.c. separatory funnel.

Extract twice with 50 and 30 c.c. of ether, respectively.

Shake the combined ether extracts with 3 c.c. of acetate buffer pH 5.0 to remove any adhering brain substances.

Discard the water layer.

Add 10 c.c. of 0.5 N sodium hydroxide solution to the ether and shake for 1 minute.

Allow the layers to separate until the alkaline layer is clear.

Run off the clear alkaline layer and analyze in the Beckman spectrophotometer.

Compare the alkaline solution with a reference solution of 0.5 N sodium hydroxide saturated with ether.

All tissues give some absorption at all wave lengths. It is necessary, therefore, to prepare blank curves on normal tissue. This is especially true when the tissues are not fresh or if they contain much fat.

Goldbaum has prepared average blank values at 255 mμ for fresh tissue.

TABLE 223

1 C.C.	OPTICAL DENSITY (MEAN OF A NUMBER OF DETERMINATIONS)	STANDARD DEVIATION FROM THE MEAN
Blood	0.045	±0.010
Liver	0.099	±0.026
Kidney	0.079	±0.020
Muscle	0.050	±0.022
Brain	0.12	±0.026

Experimental.—Rabbits weighing approximately 3 kilograms were injected intravenously or intraperitoneally with four different barbiturates representing various degrees of duration of action. Blood samples were withdrawn by cardiac puncture, at different time intervals. The rabbits were killed by air embolism and the distribution of the barbiturates in the tissues was determined. The results are given in Table 223.

Phenobarbital, a long-acting barbiturate, shows a blood level which falls very slowly; it remains at a high level 345 minutes after the injection of the drug. With the short-acting barbiturates, the blood levels fall at a more rapid rate. Disappearance of the barbiturates from the blood is related to the duration of their action; i.e., Amytal falls at a slower rate than Seconal. No evidence of cyclic behavior in barbiturate blood levels was found. Concentration of barbiturates was found to be highest in the liver and lowest in muscles.

This procedure is applicable to both pharmacologic and toxicologic investigation of barbiturates.

DETECTION AND DIFFERENTIATION OF BARBITURATES AND OTHER SEDATIVE DRUGS

Motley[2] describes the following procedures to differentiate opiates and barbiturates.

Test Reagents.—

Mercurous Nitrate.—15 per cent solution in 5 per cent nitric acid. A few drops of mercury must be kept in the bottom of the bottle.

Potassium Iodide.—N/1 (16.5 per cent) aqueous solution.

Mercuric Nitrate.—15 per cent solution in 5 per cent nitric acid.

Ferric Chloride.—N/1 (9 per cent) aqueous solution.

All of the test reagents must be kept in amber-colored bottles and must be discarded when they become hazy or turbid. Hard tap water (as Columbia, Mo., deep well water) may be used as a solvent for test materials with mercuric nitrate but is quite incompatible with mercurous nitrate, as are also hot water and alkalis.

Mercurous Nitrate Test

The addition of from 5 to 25 mg. of soluble barbiturate to 10 c.c. of water in a clean test tube containing 0.04 c.c. or one drop of the mercurous nitrate test solution produces a whitish to gray gelatinous or flocculent precipitate (Motley). *Sodium barbital* and *Pentothal sodium* form a finer precipitate with less tendency for flocculation. The barbiturate-mercurous nitrate precipitate is insoluble in acetic acid but soluble in nitric and sulphuric acids. The flocculent character of the precipitate is more marked when the pH is below 7.0, although the reaction occurs within a pH range from 4.5 to 9.5 as determined on a Beckman pH meter. The addition of a small amount of acetic acid does not interfere with the test and causes the precipitate to be more gelatinous. A large excess of barbiturate tends to dissolve some of the precipitate and make it less gelatinous. Alcohol up to 20 per cent may be used to dissolve drugs that are only sparingly soluble in cold water.

The addition of 0.08 c.c. or two drops of potassium iodide test solution to the barbiturate-mercurous nitrate precipitate produces a greenish color by reflected light and the character of the precipitate changes from gelatinous to one with no visible particles. On standing. the precipitate slowly settles, some sticking to the sides of the tube, and the greenish color by reflected light becomes yellow-green, somewhat like a blank. The reagents lose the green color most rapidly with phenobarbital of any of the barbiturate group, although, usually, a greenish tinge is perceptible after one or two hours. In the case of barbital sodium, the fine precipitate that settles out has a definite green color, being only slightly faded. With 10 mg. or more of Pentothal sodium, a thiobarbiturate, a slaty gray precipitate forms with mercurous nitrate and on the addition of potassium iodide the color does not change to the characteristic green by reflected light. The addition of potassium iodide to a blank produces a canary yellow color by reflected light and a fine colloid-like precipitate. Spectrophotometer determinations on light transmission from 400 to 700 angstroms revealed specific differences between the control and a sample with barbiturate.

As can be seen from Tables 224 and 225, this test is useful in the quick differentiation of the barbiturates from other sedative drugs, particularly the opiates; *dilantin sodium, theobromine,* and *theophylline* compounds give similar test and cannot be eliminated by these reactions. *Caffeine* compounds, *sodium benzoate,* and *sodium salicylate* produce the typical gelatinous precipitate with mercurous nitrate but on the addition of potassium iodide a coarse yellow precipitate results. Table 226 shows that the addition of mercurous nitrate alone identifies *aminopyrine* from *antipyrine* and these two in turn from *acetanilid*

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[2] J. Missouri M. A., July, 1941.

TABLE 224.—MERCUROUS NITRATE

Precipitate Gelatinous to Flocculent	
Barbiturates	+ KI = Exceedingly Fine Greenish Precipitate
Dilantin sodium	
Theobromine compounds	
Theophylline compounds	
Caffeine compounds	+ KI = Yellow Coarse Precipitate
Sodium benzoate	
Sodium salicylate	
Precipitate Non-Gelatinous	
Apomorphine (pinkish tinge)	
Bromides	+ KI = Yellow to Dark Green Coarse Precipitate
Chlorides	
Hydrochlorides	
Phosphates	
Precipitate Very Small Amount or Opalescent	
Theophylline alkaloid	
Aminophylline	
Malonic acid	
Antipyrine	
No Precipitate	
Acetanilid	
Acetophenetidin	
Acetylsalicylic acid	
Aminopyrine	
Carbromal	
Chloral hydrate	
Sulphonmethane	
Sulphonethylmethane	
Caffeine alkaloid	
Theobromine alkaloid	
Sulphates	
Urea	

TABLE 225.—MERCURIC NITRATE

Precipitate Gelatinous to Flocculent	
Barbiturates	$FeCl_3$ = Clear Amber
Dilantin sodium	$FeCl_3$ = Ppt. Present
Caffeine sodiobenzoate	$FeCl_3$ = Brown Yellow Ppt.
Theobromine and theophylline salicylate compounds	$FeCl_3$ = Dark Purple
Sodium benzoate	$FeCl_3$ = Ppt. Present
Precipitate Fine Small Amount to Opalescent	
Other caffeine compounds	
Codeine phosphate	
Aminophylline	
No Precipitate	
Morphine sulphate (greenish tinge)	
Dilaudid hydrochloride (bluish color with $FeCl_3$)	
Acetanilid	
Acetphenetidin	
Aminopyrine (dark purple fades with $FeCl_3$)	
Antipyrine (wine red with $FeCl_3$)	
Acetylsalicylic acid	
Bromides	
Carbromal	
Chloral hydrate	
Sulphonmethane	
Sulphonethylmethane	
Malonic acid	
Caffeine alkaloid	
Theobromine alkaloid	
Theophylline alkaloid	
Apomorphine (light red color clear)	

TABLE 226.—DIFFERENTIATION OF BARBITURATES FROM OTHER DRUGS

Drug	$HgNO_3$	KI	$Hg(NO_3)_2$	$FeCl_3$	KI
Barbital, phenobarbital, pentobarbital, Amytal, Alurate, Evipal (sodium)	White-gray gel. ppt.	Green fine ppt.	White gel. ppt.	Clear	Clear
Ortal sodium	White gel. ppt.	Green fine ppt.	White gel. ppt.	Ppt. present	Hazy
Seconal	White gel. ppt.	Green fine ppt.	White gel. ppt.	Hazy	Hazy
Pentothal sodium	White-gray fine ppt.	Green-gray fine ppt.	White gel. ppt.	White fine ppt.	Clear
Dilantin sodium	White gel. ppt.	Green fine ppt.	White gel. ppt.	Ppt. present	Ppt. present
Aminopyrine	Purple (fades)	Green coarse ppt.	Clear	Purple (fades)	Clear
Antipyrine	Gray fine ppt.	Slate gray ppt.	Clear	Wine red	Yellow coarse ppt.
Acetanilid					
Acetphenetidin	Clear	Yellow fine ppt.	Clear	Clear	Clear
Sulphonmethane					
Sulphonethylmethane	Clear	Gray fine ppt.	Clear	Clear	Clear
Potassium and sodium bromide	White fine ppt.	Yellow fine ppt.	Clear	Clear	Clear
Sodium salicylate	Light gray gel. ppt.	Yellow coarse ppt.	White gel ppt.	Dark purple	Clear
Aspirin, Avertin, chloral hydrate	Clear	Yellow fine ppt.	Clear	Clear	Clear
Carbromal	Clear	Dark brown ppt.	Clear	Clear	Clear
Scopolamine HBr.	White fine ppt.	Yellow coarse ppt.	Clear	Clear	White fine ppt.
Heroin	White fine ppt.	Brown coarse ppt.	Clear	Clear	Gray ppt.
Morphine sulphate	Clear	Green coarse ppt.	Clear-green	Clear-green	Clear-green
Apomorphine HCl.	White ppt.-pink	Gray fine ppt.	Clear-red	Red turbid	Yellow turbid
Codeine phosphate	White fine ppt.	Green coarse ppt.	White fine ppt.	Clear	Yellow ppt.
Codeine sulphate	Clear	Green coarse ppt.	Clear	Clear	Yellow ppt.
Dilaudid HCl.	White fine ppt.	Green fine ppt.	Clear	Clear-blue	White ppt.
Papaverine HCl.	White fine ppt.	Brown coarse ppt.	Clear	Clear	White ppt.
Strychnine, benzedrine					
Atropine (sulphate)	Clear	Green coarse ppt.	Clear	Clear	Clear
Cocaine HCl.	White fine ppt.	Green coarse ppt.	Clear	Clear	White coarse ppt.

and *acetophenetidin* which give no reaction with the reagent. *Avertin, carbromal, chloral hydrate, sulphates, sulphonmethane,* and *sulphonethylmethane* do not produce a precipitate with mercurous nitrate. *Morphine* and *codeine* with both reagents produce a greenish coarse precipitate, which settles quickly, and makes an easy distinction from the barbiturates. *Apomorphine* gives a white precipitate with mercurous nitrate which develops a pinkish tinge on standing a few minutes.

Mercuric Nitrate Test

The addition of from 5 to 25 mg. of a soluble barbiturate to 10 c.c. of water in a test tube containing one drop of mercuric nitrate test solution produces a white gelatinous precipitate, similar in character to the one produced by mercurous nitrate but whiter and more flocculent. (Table 225.)

The addition of two or more drops of potassium iodide test solution dissolves the barbiturate-mercuric nitrate precipitate of those tested, except in the case of *Ortal sodium* and *Seconal* in which the precipitate only partly disappears so that the test mixture becomes hazy. Potassium iodide does not dissolve the precipitate of *Dilatin sodium* with mercuric nitrate but does dissolve that of *Pentothal sodium.* The addition of two or more drops of ferric chloride solution also dissolves the mercuric nitrate-barbiturate precipitate except for *Ortal sodium* and *Seconal* which, as with potassium iodide, fail to clear completely. Ferric chloride does not dissolve the precipitate of *Pentothal sodium* or *Dilantin sodium.*

Table 226 shows that *Pentothal sodium* and *Dilantin sodium* may be distinguished from the barbiturates by using potassium iodide and ferric chloride in conjunction with mercuric nitrate. The various *salicylate compounds of theobromine and theophylline* give a gelatinous white precipitate with mercuric nitrate and a dark purple color appears on the addition of ferric chloride. The purple color will form with ferric chloride alone but is intensified by the mercuric nitrate. The salicylate part of the molecule is responsible for this chromogenic reaction.

Morphine sulphate with mercuric nitrate imparts a greenish tinge to the solution which remains clear and is not changed by ferric chloride or potassium iodide. *Codeine phosphate* gives a fine white precipitate with mercuric nitrate while the sulphate remains clear; but both the phosphate and sulphate salts of codeine produce a yellow precipitate on the addition of potassium iodide while the use of ferric chloride in place of the potassium iodide results in a clear solution in both cases. *Dilaudid hydrochloride* does not produce a precipitate with mercuric nitrate but the addition of ferric chloride yields a clear bluish solution, the color of which persists. With mercuric nitrate alone, *apomorphine* gives a clear red solution which does not fade. The opiates, *morphine, codeine, dilaudid,* and *apomorphine* can be differentiated from one another by these tests.

Ferric chloride with *aminopyrine* develops a deep purple color which fades in less than 60 seconds; however, if mercuric nitrate is added first the color persists for two or three minutes. *Antipyrine* gives a wine red color with ferric chloride while *acetanilid* and *acetophenetidin* do not. *Acetylsalicylic acid, Avertin, bromides, chloral hydrate, carbromal, sulphonmethane* and *sulphonethylmethane* give no color reaction or precipitate with any of the three test reagents.

Test for Acetanilid and Phenacetin

Acetanilids

(1) Hydrolyze with a few drops of dilute hydrochloric acid. Heat gently. Then apply the **isonitrile test**: Add a few drops of chloroform and a few drops of 10% sodium hydroxide and heat gently. A positive reaction is denoted by production of the strong pungent skunk-like odor of isonitrile.

(2) White crystals, melting point 114° C.

Phenacetin (Acetophenetidin)

(1) White crystals, melting point 135° C.

(2) Boil for a few minutes with several drops of concentrated hydrochloric acid to hydrolyze. Add 1 c.c. of saturated solution of phenol. Add several drops of freshly prepared saturated solution of calcium hypochlorite. The pink to red color of indophenol is produced, which becomes violet when concentrated ammonia is added, if phenacetin is present.

Note: The indophenol test is positive for both acetanilid and phenacetin. Differentiation is effected by using the isonitrile test which is positive for acetanilids but not for phenacetin.

Detection and Estimation of Aspirin or Salicylates in Tissue or Plasma[1]

Principle.—

Aspirin is hydrolyzed to salicylic acid by hydrochloric acid. Or the salicylic acid is extracted from acidified plasma with ethylene dichloride, then returned as the colored ferric complex to an aqueous phase and estimated colorimetrically.

Technic.—

Shake 2 c.c. of plasma in a separatory funnel
with 0.5 c.c. of 6N hydrochloric acid
and 30.0 c.c. of ethylene dichloride.

Shake well for complete extraction of the salicylate. Allow the two immiscible layers to separate. Discard the water layer.

Save the nonaqueous layer (ethylene dichloride).

Add 10 c.c. of 0.025% ferric nitrate solution to this nonaqueous layer and shake.

If salicylates are present, a blue color will develop, the intensity of which will vary directly with the concentration of salicylate present.

Treat varying concentrations of salicylates in a similar manner and use these for comparison of the unknown.

Serum Salicylates

Method of F. Velez Orozco and F. Guerra[2]

Reagents.—

2/3 N Sulphuric Acid.—

See page 225.

10% Sodium Tungstate Solution.—

See page 225.

9% Ferric Chloride Solution.—

Dissolve 9 grams of ferric chloride in distilled water and dilute to 100 c.c. with distilled water. For use dilute 1:10 with distilled water.

Technic.—

Place 2 c.c. of blood serum in a test tube.
Add 2 c.c. of 2/3 N sulphuric acid.
Add 9 c.c. distilled water
and 2 c.c. of 10% sodium tungstate solution.
Mix thoroughly, centrifuge, then filter through filter paper.
Place 9 c.c. of the filtrate in a colorimeter tube.

Add 0.2 c.c. of dilute ferric chloride solution (1 part reagent and 9 parts of water). Mix.

Read in the Lumetron within not more than 2 minutes, using green filter 530 against water as the blank set at 100 per cent transmission. Obtain the result in mg. per cent sodium salicylate from a calibration graph or card.

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[1] Brodie, B., Udenfriend, S., Coburn, A.: J. Pharmacol. & Exper. Therap. **80**: 114, 1944.

[2] Archivos del Instituto de Cardiología de Mexico **16**: 131, 1946. Modified for adaptation to the Lumetron (Photovolt) model 400-A.

To convert these results from mg. per cent sodium salicylate to mg. per cent salicylic acid, multiply the result by 0.86.

Calibration Procedure.—

1% Sodium Salicylate Solution.—Dissolve 1 gram sodium salicylate, c.p., in distilled water in a 100 c.c. volumetric flask and dilute to volume with distilled water.

Add 1 c.c. of the standard sodium salicylate solution to 9 c.c. of pooled serum, free from salicylates (that is, from individuals known not to have taken any salicylates).

Dilute 1.0, 2.0, 3.0 c.c. of this serum salicylate solution respectively to 5.0 c.c. with salicylate-free serum. This yields sera containing respectively 20, 40, and 60 mg. per cent sodium salicylate.

Add 2 c.c. of 2/3 N sulphuric acid, 9 c.c. distilled water, and 2 c.c. of 10% sodium tungstate to each and continue as in the test.

Read the per cent transmission in the Lumetron, using green filter 530 against water as the blank set at 100 per cent transmission. If the calibration is carried out properly and if the proper reagents are used, 40 mg. per cent sodium salicylate will read somewhere in the region of 55% transmission.

Using Lumetron semi-log graph paper, assign to the concentration axis the values 0-125 mg. per cent sodium salicylate, and plot the transmission readings above the corresponding concentration values. The points will fall approximately on a straight line which intersects the transmission axis near the 100 per cent point. For the sake of convenience in use, the concentration values may be taken from the graph and entered into a Lumetron blank calibration card.

DETECTION OF MARIJUANA (CANNABIS SATIVA, LINN)

Marijuana is of great interest in many quarters due to the fact that its use by drug addicts is spreading in this country.

This plant grows wild along the roadsides in many states, particularly in the West. The proper name of the plant is *Cannabis sativa.* Marijuana is a mongrel Mexican word, meaning "pleasant feeling." In underworld argot, the drug is known by many different names, such as, "Indian hay," "greefo," "mu," "mooter," "reefer," and "Mary Warner." Yawger[1] has given a very complete description of this drug and its relationship to crime. He states that the Arabic word "hashishan" is responsible for our word assassin. In Persia, there lived a military and religious sect known as the Assassins, who were sometimes incited to greater violence in their atrocities by the use of hashish which is the historic name of marijuana. Hashish or marijuana was cultivated in China three centuries before Christ. It is alluded to in the Assyrian, Greek, and Roman writings. Napoleon's soldiers were forbidden to use it in Egypt in 1800. The use of hashish is common among the natives of India, Egypt, and North Africa.

The effects of this drug as it is commonly used are obtained through the medium of smoking cigarettes made from dried parts of the plant and leaves. The odor given off by the cigarette smoke is characteristic and distinctive. Smoking produces euphoria. John Stuart Mill, the philosopher, once wrote of its power to revive forgotten memories. Smokers will tell you that under its influence they are able to recall events long since forgotten.

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[1] Am. J. Sc. **195**: 351, 1925.

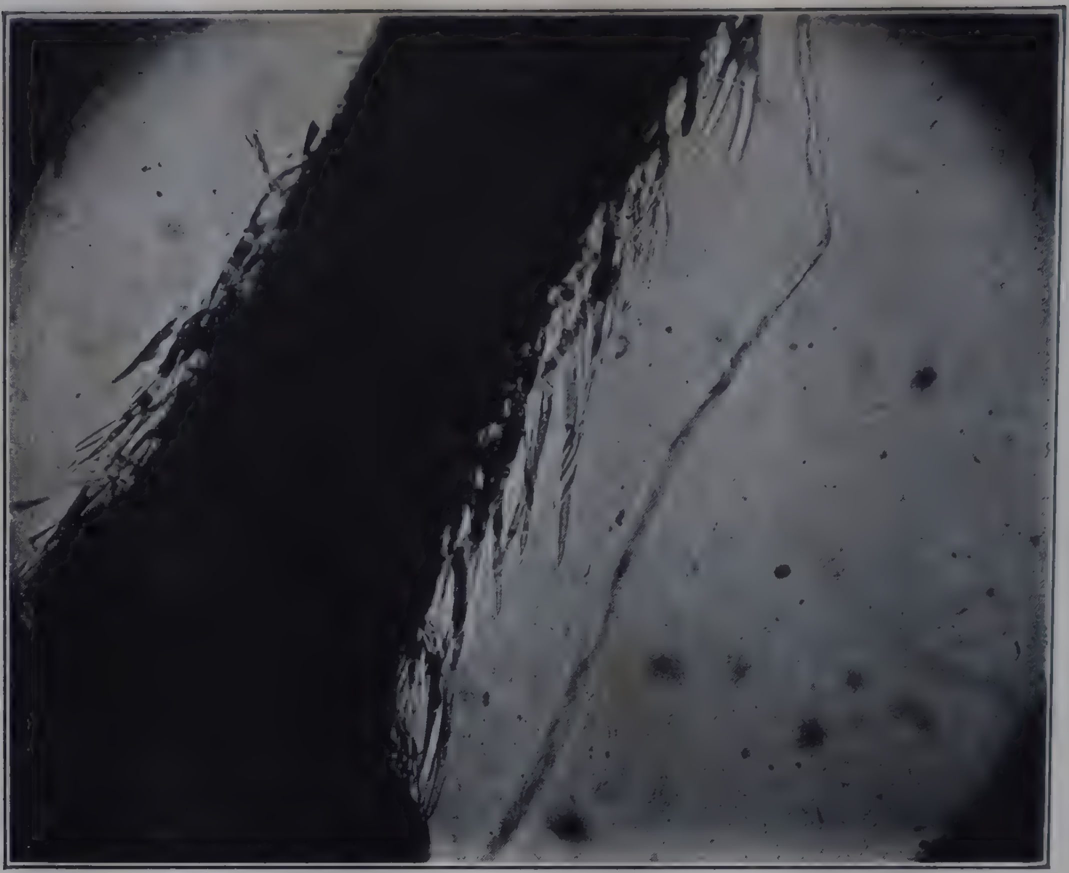

Fig. 700.—Stem of a marijuana plant. (About 75×.) Specimen found mixed with tobacco in the purse of a woman alleged to have committed suicide apparently under the influence of marijuana.

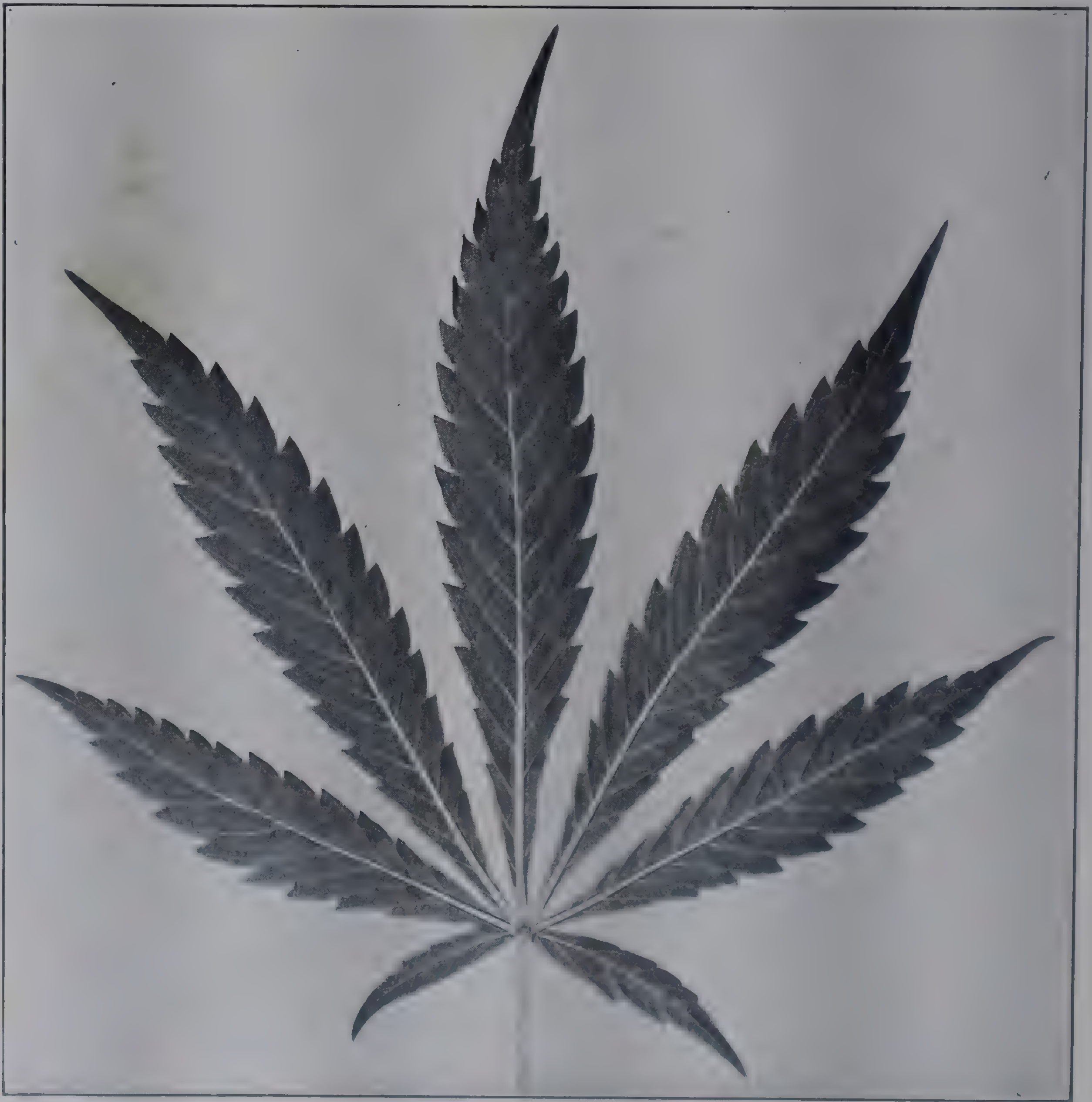

Fig. 701A.—Leaf of marijuana plant. (Courtesy Mr. H. J. Anslinger, Commissioner, Bureau of Narcotics, Treasury Department, Washington, D. C.)

The first noticeable change in the smoker is a lessening of his power to control his thoughts and actions fully. There are rapid interruptions in the continuity of thought. In addition to euphoria and exaltation, the smoker is aware of increased power and energy. Several Mexican addicts assert that they smoke the weed at night after a hard day's labor to secure relaxation. Only by determined effort of the will is the smoker able to withdraw from his reveries, but if there be further indulgence, such possible lucid intervals become shorter and less frequent. Illusions are common and faces of others often take on grotesque expressions. Imagination may be stimulated to the

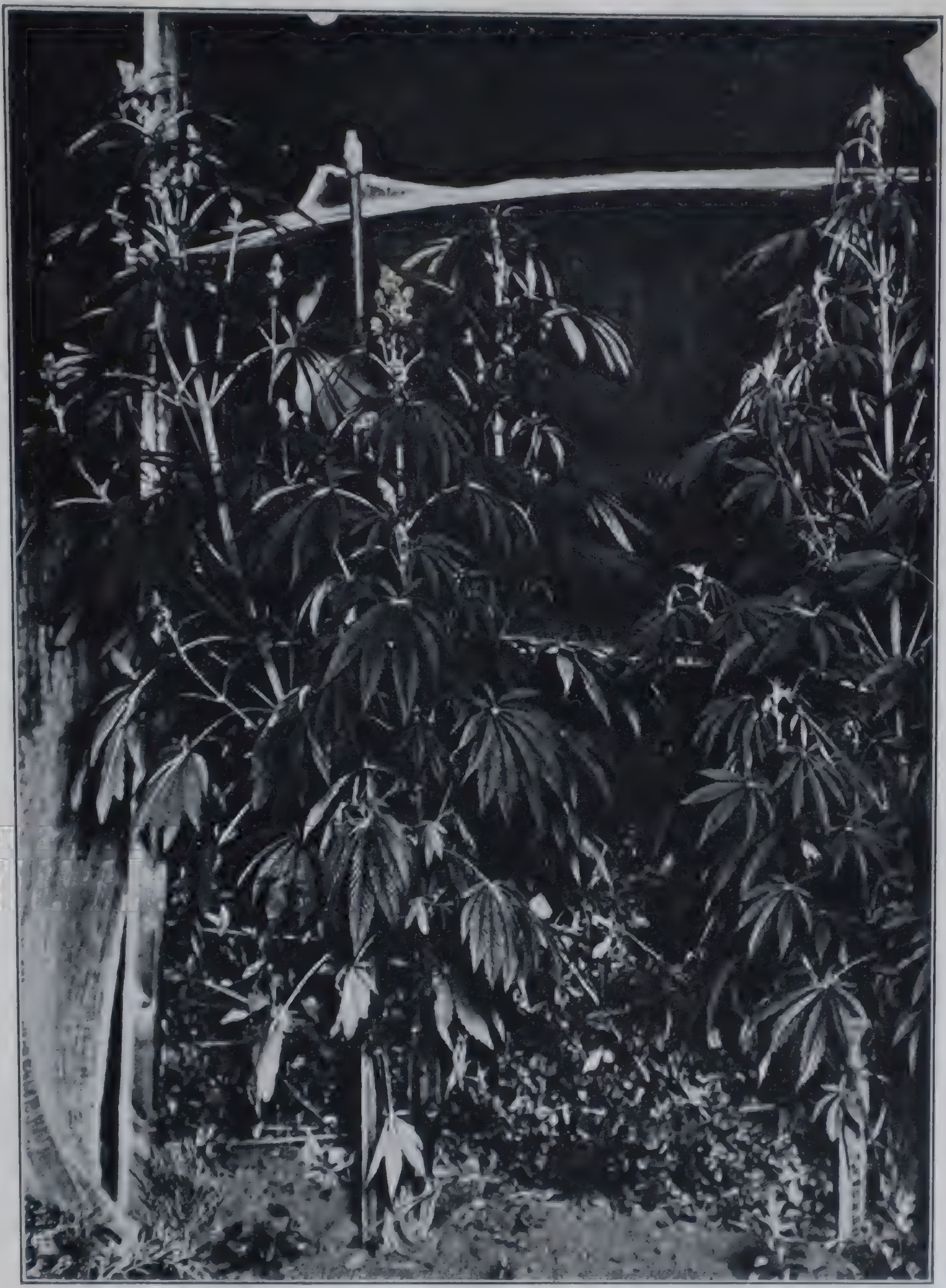

Fig. 701*B*.—Marijuana, flowering plant. These plants may grow more than six feet high. (Courtesy Mr. H. J. Anslinger, Commissioner, Bureau of Narcotics, Treasury Department, Washington, D. C.)

extent of delirium, sometimes with hallucinatory creations appearing. The effect may be pleasing, ludicrous, gruesome, or sensual. Dominant emotions become magnified—the genial man grows fonder of his fellows, the quarrelsome, more pugnacious, the timid more fearful, and the criminal emboldened. Exaggerations make the plainest food delicious. The hearing may be painfully intensified. Perception is disturbed in the matter of time and space; minutes seem hours and hours days; a long stride or a jump may be taken to step over a pebble or small stick.

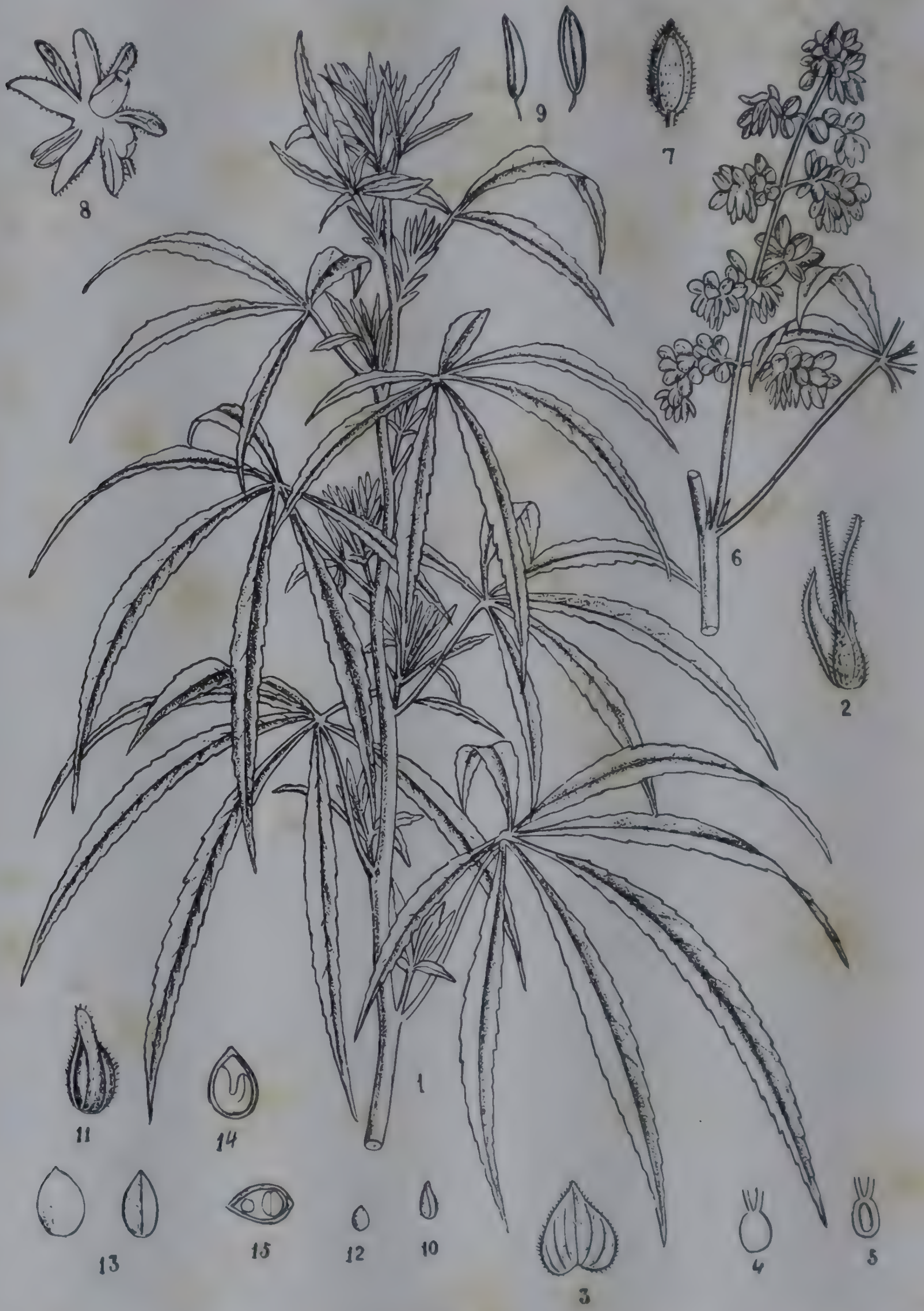

Fig. 702.—*Cannabis sativa.* Linn. (Drawn from specimens grown in Kew Gardens.) *1.* Upper part of a female plant. *2.* A female flower. *3.* Perianth unrolled. *4.* Ovary. *5.* Section of the same. *6.* A male panicle. *7.* A bud. *8.* A male flower. *9.* Stamens. *10, 11.* Fruit surrounded with the persistent perianth. *12, 13.* Fruit. *14.* Vertical, and—*15.* Transverse section of fruit. (Medicinal Plants by Robert Bentley and Henry Trimen. Vol. IV, Nos. 228-306, London.)

Relation to Crime.—It is generally believed that the use of this drug will cause chronic mental deterioration, an effect that has been noted for ages in India and Egypt.

Marijuana, in other words, is an American hashish and its effects correspond to those described in the literature of the hashish smoker of the Orient.

Fig. 700 is a photomicrograph of the stem of a marijuana plant, magnified about 75 times. Note the hooklike processes along both sides of the stem. This marijuana was taken from a specimen mixed with tobacco found in the purse of a woman who was alleged to have committed suicide, apparently under the influence of marijuana.

The identification of marijuana becomes necessary in view of the widespread use of the plant in cigarettes in this country. Marijuana comes within the bounds of the narcotic law and its use is also interdicted in a number of states by special legislative acts. It is a habit-forming drug and produces serious effects in addicts. It can be identified by microscopic examination. Fig. 701*A* shows the leaf and Fig. 701*B* the flowering plant which grows very tall. Fig. 702 is a plate drawn from specimens grown in Kew Gardens. These cuts are made from photographs furnished through the courtesy of Mr. H. J. Anslinger, Commissioner, Bureau of Narcotics, Treasury Department, Washington, D. C.

In the case of extracts and similar preparations containing marijuana, it is necessary to use the assay described in the tenth revision of the U. S. Pharmacopoeia under the monograph for cannabis.

Chemical Tests for Marijuana

Of all the methods of testing samples of material suspected of being marijuana, microscopic examination is generally the most satisfactory. An experienced observer should never be misled even though the marijuana present in the sample is only a small portion of the whole. The method is completely described in the Treasury Department publication *Marihuana—Its Identification.**

It is generally desirable to supplement microscopic examination by one or more chemical tests. Of the tests known at present only one is completely reliable, the modified Duquenois test. This test was first described by M. Pierre Duquenois and Hassan Negm Mustapha.[1] The test has not been shown specific for marijuana, but on the other hand no sample of marijuana has thus far been encountered which failed to respond. Certain samples of tobacco have been found to give a color response which might be confused with the color response of marijuana. The color response of marijuana may, however, be readily differentiated from that of tobacco by virtue of the different solubility characteristics of the colored product. The purple produced in the test by marijuana is readily extracted from aqueous solution by chloroform, while the color produced by tobacco is not extracted, but remains in the aqueous layer. The original test has, therefore, been modified to include this differentiation.

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*For sale by the Superintendent of Documents, Government Printing Office, Washington, D. C., price 10 cents.

[1] J. Egyptian M. A. 21: 224, 1938; Chem. Abstracts 32: 5993, 1938.

Reagents.—

Duquenois Reagent.—

To 20 c.c. of 95% alcohol
add 5 drops of acetaldehyde
and 0.4 gm. of vanillin.

This reagent may be kept for some time in a glass-stoppered bottle, preferably in a refrigerator or in a cool, dark place. It should be discarded after the development of a deep yellow color.

Activated Charcoal.—

This is obtained as Darco, Norit, or similar material.

Petroleum Ether.—

30° to 80° C., B.P.

Hydrochloric Acid.—

Use the concentrated reagent grade.

Chloroform.—

Use the reagent grade.

Technic.—

Extract 0.5 gm. of bulk material or 2 cigarettes in a test tube with petroleum ether. Cigarettes need not be broken open. Allow about 1 hour for the extraction.

Add about 1 gm. of activated charcoal, shake, and centrifuge or filter.

Evaporate the filtrate in a white porcelain dish.

Add 1 c.c. of Duquenois reagent to the residue and swirl to dissolve.

Add 0.5 c.c. of hydrochloric acid and swirl again.

The presence of marijuana is indicated by a transitory series of colors: green, slate, and finally indigo-violet.

Transfer the solution to a test tube and add 2 c.c. of chloroform and shake.

The indigo-violet color will be transferred to the chloroform layer, if this is marijuana.

The Beam Test

Attach a suspected cigarette to a small test tube, or cylinder, with an ordinary paper clip, and with the aid of a small funnel pour 5 c.c. (1 thimble full) of petroleum ether into one end of the cigarette so that the liquid flows through the interior of the cigarette, extracting the filler on its way.

1. Pour about one-half of this extract into a small white porcelain dish and evaporate, add to the evaporated portion a few drops of Solution No. 1 (1% potassium hydroxide in alcohol). The residue in the dish should become purple in a minute or so, the color deepening gradually on standing.

—OR—

2. Add to the remainder of the solution in a test tube about 1 c.c. of Solution No. 2 (absolute alcohol saturated with hydrochloric acid gas) and shake; allow to settle. The lower layer should be distinctly pink.

The microscopic examination, as stated above, is possibly the most important part of the examination. The cellular hairs and cystoliths on the plant are very characteristic. If hydrochloric acid is added to the plant, bubbles are seen to escape microscopically due to the presence of calcium carbonate.

Bioassay in Animals

Bioassay in Dogs.—The degree of incoordination produced in dogs is the most common method for estimating biologic activity. The dogs at first are quieted and there is a characteristic expression suggestive of apprehension. Then a swaying of the body develops, which becomes progressively more intense, the direction of swaying being backward and forward as well as from side to side. The dogs try to stand in a characteristic position with the feet spread apart, to prevent them from toppling over. When placed in abnormal positions, the animals make no effort to correct the position. It has been said by Walton,[1] who has written an excellent book on this subject, entitled *Marihuana*, that a characteristic feature of the intoxication is the suddenness with which they can be aroused to an almost normal state of activity by some special stimulus. After the exciting stimulus, they return to the earlier depressed condition.

With large doses, the dogs show fibrillary tremors. Vomiting and retching occur; persistent scratching, uncompleted walking movements are observed; the head sinks lower and lower and is suddenly jerked back, only to sink again. Fraenkel compared this performance to a person dozing on a train, where he is frequently stimulated to a state of partial wakefulness and immediately dozes back.

Walton, Martin, and Keller defined six arbitrary stages of intensity of effects: First, a slight decompression; second, a barely recognizable ataxia; third, an obvious ataxia; fourth, a marked ataxia in which the animal frequently pitches forward and barely catches itself; fifth, inability to stand alone; sixth, inability to rise and plunge about.

Bioassay in Rabbits.—Rabbits do not exhibit swaying movement to as marked degree as dogs. They are usually made quiet by the drug. Walton, Martin, and Keller used the so-called von Frey hairs which elicit the wink reflex when pressed against the cornea of normal rabbits but would have no effect in narcotized animals. These men used von Frey hairs which bent at a pressure of 30 to 50 mg. The number of wink responses obtained with 20 strokes of the hair against the cornea was taken as a measure of the degree of anesthesia. They state that rabbits responded to about 16 of the 20 stimuli; with moderate doses the response would drop to about eight in twenty, and with larger doses would disappear altogether.

Bioassay in Rats and Mice.—Recognizable effects are seen in mice with 0.2 to 0.4 mg. of the vacuum distillate according to Walton, Martin, and Keller's method.

DETECTION OF OXALIC ACID

A solution of oxalic acid or of potassium binoxalate reddens litmus paper.

Either of these substances, with excess of calcium hydroxide, acetate, or sulphate, gives a white precipitate of calcium oxalate, which is insoluble in ammonia or acetic acid, but soluble in strong hydrochloric or nitric acid.

Vomited matter from a case of oxalic acid poisoning should be searched for the leaves of sorrel or green material of the rhubarb pie. This is to exclude the possibility of a complication in the analysis. In gastric contents, oxalic acid will be partly free and partly combined as the insoluble calcium or magnesium oxalates. For the free oxalic acid, the following method must be used:

1. Having made an extract with hot dilute hydrochloric acid and having filtered it, add lead acetate, which precipitates the lead oxalate along with various other lead compounds. Suspend this deposit in water and pass hydrogen sulphide through it for two hours. The oxalic acid is set free, the black lead sulphide being thrown down. Filter and test the filtrate with calcium acetate.

2. If the oxalic acid is in the combined state, use the following method: Digest the suspected material with warm dilute hydrochloric acid until the mixture is quite fluid, filter, and to the filtrate add ammonium hydroxide until an alkaline reaction is reached. Let stand, decant, and collect the deposit on a filter. This deposit is calcium oxalate. The filtrate is mixed with the decanted fluid, is treated with excess of calcium acetate, and the precipitate separated on a filter. This second deposit represents the free acid in the original material. Determine the nature and amount of the first deposit by washing with acetic acid on the filter and afterward putting into a beaker and dissolving by cautiously adding strong hydrochloric acid and gently heating. Excess of ammonia will precipitate it completely if sufficient time is allowed. After decanting the clear fluid wash the deposit by decantation, put into a tared dish, dry in a water bath, and weigh.

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[1] Walton, Robert P.: Marihuana. J. B. Lippincott Co., 1938.

If this deposit is calcium oxalate, it will be white, and, when a portion is heated on platinum, leave a gray ash of calcium carbonate. Another portion warmed in a test tube with strong sulphuric acid evolves carbon dioxide gas, which can be identified by conducting it through a delivery tube into baryta-water. A third portion, suspended in water slightly acidulated with sulphuric acid, will discharge the purple color of potassium permanganate. This test can be applied by standard volumetric solutions and an estimation of quantity obtained.

GROUP III. ALKALINE-ETHER EXTRACTION

Use the residual aqueous solution from the acid-ether extraction, Group II. Alkalinize slightly with sodium hydroxide solution. Again extract with several portions of ether. Collect these extracts in a small evaporating dish and place them on a steam bath to drive off the ether.

The residue may be used for testing many poisons of the alkaloid group. The morphine group, however, is not soluble under the conditions of this extraction. To obtain the morphine group, neutralize the residual aqueous solution, above, with dilute hydrochloric acid and then alkalinize with *ammonia.* Extract with several portions of *ethyl acetate,* collect the extracts, and evaporate to dryness on a steam bath.

Purify these residues either by using selective solvents or by sublimation. Remember that with the presumptive tests many of the protein impurities give false positive reactions and may therefore be confused with true alkaloids. Further tests are indicated in such cases.

Reagents for Alkaloid Precipitation.—

Wagner Reagent.—

Dissolve 10 gm. potassium iodide, c.p.
and 1 gm. iodine, resublimed
in 100 c.c. of distilled water.

Mayer Reagent.—

Dissolve 1.4 gm. mercuric chloride, c.p.
and 5.0 gm. potassium iodide, c.p.
in 100.0 c.c. distilled water.

Marme Reagent.—

Dissolve 20 gm. potassium iodide, c.p.
in 20 c.c. of hot distilled water.
Add 10 gm. of cadmium iodide, c.p.,
and dilute to 100 c.c. with distilled water.

Picric Acid.—

Saturated solution of picric acid.

Platinic Chloride Reagent.—

Dissolve 5 gm. platinic chloride in distilled water and dilute to 100 c.c. with distilled water.

Technic for Precipitate Tests.—

Dissolve the residue in a minimum amount of very dilute acetic acid (1:100).
Place 1 drop of this dissolved residue on a glass slide.
Add 1 drop of precipitating reagent.
The various alkaloids yield typical crystals.
Protein products of putrefaction may interfere with this test.

Color Reactions.—

The following color reactions, which are usually more specific than the precipitating reactions, are made on the original dry residue.

Scrape off a small portion of residue and place it on a glass slide.

Carry out the color reaction from here using the proper reagent. These reactions are given in detail in Table 227.

Most of the chemical tests for alkaloidal poisons are color reactions, some are physiologic or biologic tests, and others are based upon the finding of specific crystals formed by specific precipitants.

There are many reagents used in the various color tests for alkaloids. The following list of color reactions is taken from Peterson, Haines, and Webster.[1]

1. **Concentrated Sulphuric Acid.**—No color, or only a faint straw shade is given by pure aconitine (which is rarely obtained), atropine, cocaine, codeine, scopolamine, hyoscyamine, gelsemine occasionally shows a yellow followed by brown (morphine often light red if reagent is impure), nicotine, quinine, quinidine, and strychnine.

Yellow, orange, or brown colorations are yielded by berberine, colchicine, delphinine, gelsemine, and lobeline. Yellow tints followed by red or violet are noted especially with the alkaloids of veratrum.

Red, purple, or violet colors are obtained with impure aconitine, apomorphine, brucine (due to impurity of nitric acid in the reagent), impure cocaine, coniine, impure gelsemine, narcotine, and physostigmine.

2. **Concentrated Nitric Acid.**—No coloration is noted with pure aconitine, atropine, cinchonine, coniine, gelsemine (may show yellow or green if impure), quinine, and strychnine.

Yellow tints are obtained with impure aconitine, berberine, codeine, gelsemine, morphine (may show red), narcotine, and veratrine.

Red, purple, or violet colors are given by impure aconitine, apomorphine, berberine, brucine (deep red), and physostigmine.

3. **Fröhde's Reagent.**—This is prepared by dissolving 1 or 2 mg. of molybdic acid in 1 c.c. of chemically pure sulphuric acid. Other directions specify higher proportions of molybdic acid, but these lessen the distinctiveness of the test.[2] This reagent must be freshly prepared just before use as it is gradually reduced on standing with production of colored oxides of molybdenum. While this reagent gives colorations with many alkaloids, its most distinctive reaction is with morphine.

No color is produced with atropine, cinchonine, coniine, hyoscyamine, nicotine, scopolamine, and strychnine. Yellowish colorations are observed with aconitine and colchicine. Reddish shades are noted with brucine and veratrine. Bluish colors are given by codeine and morphine (purple fading to violet, then dirty green, yellow, and finally to faint pink). Greenish shades are yielded by apomorphine, berberine, and quinine.

DETECTION OF MORPHINE IN ANIMAL TISSUES

(A) Separation from the Tissues

The entire specimen should be used. Weigh the material before testing.

Cut the viscera into fine particles.

Place in a flask to which is connected a reflux condenser, and cover with 50% alcohol distinctly acidulated with acetic acid (or if there is a possibility of conversion of morphine to apomorphine, use hydrochloric or salicylic acid).

Digest in the closed flask at 70° C. for one hour.

Cool.

Strain through gauze.

Wash the residue with a little of the dilute acidulated alcohol.

Return to the flask.

Repeat the digestion and straining of the residue for a second and a third time.

Evaporate the mixed alcoholic filtrates and washings in the water-bath to a syrupy liquid.

Add to this three times its volume of very slightly acidulated (acetic) alcohol, 95%.

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[1] Legal Med. & Toxicol. 2: 427, 1923.

[2] Fröhde's original communication (Ann. Chem. **120**: 188, 1861) called for 0.01 gram of sodium molybdate in 1 c.c. of concentrated sulphuric acid. See, however, Arch. Pharm., **176**: 54, 1866.

TABLE 227

Reactions of the More Important Alkaloids and Glucosides*

ABBREVIATIONS

Bl = blue
bl = bluish
Bk = black
bk = blackish

Br = brown
br = brownish
Ca = carmine
ch = cherry

Cr = crimson
dk = dark
dt = dirty
exs = excess

G = green
g = greenish
In = indigo
lm = lemon

lt = light
ol = olive
Or = orange
ppt = precipitate

P = purple
R = red
r = reddish
Sc = scarlet

soln = solution
V = violet
Y = yellow
y = yellowish

Wh = white
wh = whitish
— = to
△ = changing to

	COLOR REACTIONS							PRECIPITATES				Various Other Reactions
	Conc. H_2SO_4	Conc. HNO_3	H_2SO_4 + HNO_3 (Erdmann)	H_2SO_4 + Ammon. (or Sod.) Molybdate 100 : 1–5 (Fröhde)	H_2SO_4 + Ammon. meta Vanadate 200 : 1 (Mandelin)	H_2SO_4 + Selenous Acid 200 : 1 (Mecke)	H_2SO_4 + Formaldehyde 25 : 1 (Marquis)	$HgI_2 . 2KI$	$BiI_3 . 4KI$	I . KI	Picric Acid	
Aconitine	colorl. pure; r impureΔrBr by heat	colorl—rBr	colorlΔV	colorl—YΔyBr	lt Br—Or	alm. colorl—y Δlt Br by heat	colorl	Wh. ppt in dil. soln	Y (1 : 40,000)	Br	ppt only in conc. soln	H_2SO_4 + sugar = R color $FeCl_3$ = Y ppt $K_2Cr_2O_7$ = Y ppt
Apomorphine	colorl—Br—R	P—Or	R—Or	GΔV	GΔBl	VΔdk Br by heat	blood RΔVRΔ yBr by heat	Y oily pptΔ cryst	1 : 10,000	blood R	Y	$FeCl_3$ = RΔBk ppt Tannin = yG ppt
Atropine	colorl	colorl	colorl	colorl	RΔY	colorl	brΔdtG by heat	1 : 7000	rYΔcanary Y (1 : 10,000)	rBr (1 : 3000)	Y only in conc. soln (1 : 200)	H_2SO_4 + $K_2Cr_2O_7$ + heat = bitter almond odor.—HNO_3 & evap.: Y res. + alcoh KOH = VΔch R
Bebeerine	brΔr by heat	R—rBr	Br	VΔrBrΔdt G	dt GΔBr		colorl—br	Wh. amorph	Or R	Br	Y amorph (1 : 3000)	
Berberine	ol GΔYΔol G by heat	rBr	olGΔyBr	br GΔol G by heat	dk Br	gY—brV—R		yG	OrR (1 : 5000)	rBr	lt Y (1 : 3000)	HCl soln + Cl water = R color H_2SO_4 + oxidizers (e.g. MnO_2) = play of colors (like strychnine)
Brucine	colorlΔY by heat	blood RΔorY	RΔY	RΔYΔcolorl	yΔOr	yRΔlmY by heat	lt R	wh Y (1 : 5000)	rY (1 : 5000)	Br (1 : 50,000)	Cryst (1 : 10,000)	H_2SO_4 + $K_2Cr_2O_7$ = OrΔR by dil. acids $K_4Fe(CN)_6$ = Y pptΔBl
Caffeine	colorl	colorl	colorl	colorl	colorl	colorl		none	none?	dt dk Br in strong soln	none or only in conc soln	Evap. w. HNO_3; res. + NH_4OH (caut.!) = P color (murexid test)
Cinchonidine	colorl	colorl	colorl	colorl	colorl	colorl	colorl					H_2PtCl_6 = lt Or ppt $AuCl_3$ HCl = Y ppt $K_4Fe(CN)_6$ = rY ppt
Cinchonine	colorl	colorl	colorl	colorl	colorl	colorl	colorl	Wh (1 : 75,000)	brick R (1 : 50,000)	dk Br (1 : 500,000)	Y (1 : 100,000)	$K_4Fe(CN)_6$ = y Wh ppt Tannin = y Wh ppt Cl-water = no color; add NH_4OH = Wh ppt
Cocaine	colorl pure, r impure	colorl	colorl	colorl	Or	colorl Δlt RΔY by heat	colorl	1 : 140,000		Br (1 : 50,000)	Y (1 : 1000)	H_2SO_4 + heat = vapors of benzoic acid H_2SO_4 + a periodate + gentle heat = bright green colorΔBlΔV $AuCl_3 . HCl$ = lt Y ppt

Reactions of the More Important Alkaloids and Glucosides—*Continued*

	COLOR REACTIONS							PRECIPITATES				VARIOUS OTHER REACTIONS
	Conc. H_2SO_4	Conc. HNO_3	H_2SO_4 + HNO_3 (Erdmann)	H_2SO_4 + Ammon. (or Sod.) Molybdate 100: 1-5 (Frohde)	H_2SO_4 + Ammon. meta Vanadate 200: 1 (Mandelin)	H_2SO_4 + Selenous Acid 200: 1 (Mecke)	H_2SO_4 + Formaldehyde 25: 1 (Marquis)	$HgI_2.2KI$	$BiI_3.4KI$	I.KI	Picric Acid	
Codeine	colorlΔBl by heatΔR by HNO_3	Y—R	colorlΔBl by heat	dt GΔBlΔlt Y	gBlΔBl	GΔBlΔol G	Ca RΔVBlΔy Br	1 : 50,000	y R (1 : 30,000)	Br (1 : 60,000)	Y (1 : 250)	$H_2SO_4 + FeCl_3 =$ deep Bl $H_2SO_4 +$ sugar = R $H_2SO_4 + K_2Cr_2O_7 =$ ol G
Colchicine	YΔR by heat	VΔbr RΔOr by NaOH	BlΔR by KOH	YΔyG	bl GrΔBr	lmYΔyBr by heat	Y	only in conc. soln	1 : 3000	Br (1 : 2500)	only in conc soln	$H_2SO_4 + K_2Cr_2O_7 =$ GΔBr Cl-water = Y ppt
Coniine	colorl	y—colorl	colorl	colorlΔY	colorl	colorl	colorl—bl	Wh (1 : 8000)	Y (1 : 6000)	dk Br	only in conc soln	
Curarine	bl—R	P	V—R	V	V	lt VΔr		ppt				
Diacetylmorphine	colorl	YΔR by heat		P	ltG	G	rVΔbl V by heat					
Digitalin	Bl—rBrΔch R		lt Br	dk OrΔch RΔBr				none	only in conc soln	turb.		
Emetine	colorl—dt Br by KOH	Or Y	GΔY	colorlΔGΔBl by HCl	Br	GΔV by water	Br	Y amorph (1: 25,000)	1 : 25,000	1 : 25,000	Y amorph (1 : 25,000)	$H_2SO_4 + K_2Cr_2O_7 =$ Br
Ethylmorphine				GΔBl by heat	gYΔG by heat	G	deep Bl					
Gelsemine	colorl—yBrΔ yG	colorl—gY		yBrΔlt VR	PΔVR						Y	$H_2SO_4 + K_2Cr_2O_7 =$ VR $H_2SO_4 +$ sugar = R
Hydrastine	colorl—yΔR by heat	Or	Y—Or R	colorl—GΔBr	Ca RΔOr R	Br	gY—br				ppt	$K_4Fe(CN)_6 =$ Wh ppt
Hyoscine (Scopolamine)	colorlΔBl by heat	on evap slt Y resΔV by alcoh. KOH	colorl	colorl			Y—OrΔbr Bk by heat	yWh		oily ppt	Y	$K_4Fe(CN)_6 =$ Wh ppt
Hyoscyamine	colorl	colorl	colorl	colorl	colorl			Wh	R ppt	Or ppt	Y (melt 161—163° C)	
Lobeline	rBr			rBr				lt Y (in acid soln)		Br in acid soln	ppt	
Morphine	colorlΔP by heat	Or RΔrY	br RΔdk Br	VΔGΔbr YΔBl	rΔblV	BlΔbl GΔBr by heat	Ca RΔV	1 : 2500	yR (1 : 5000)	r Br (1 : 5000)	none	$FeCl_3 =$ Bl in neutral soln (color destroyed by acids or alcoh.) 1 : 5000
Narceine	BrΔblood R by heat	br YΔdk Or by heat	YΔbr YΔOr by heat	br GΔdk ol GΔ ch R by heat	V—Or	gY Δ V by heat	YΔrBrΔdk R by heat	amorph (1 : 10,000)	Y	Br	Y (1 : 3000)	
Narcotine	colorlΔYΔR by heat	gY—rY	rVΔR	bl GΔchR	CaR	gBlΔchR by heat	VΔblood R	Wh (1 : 50,000)	lt Y (1 : 4000)	r Br (1 : 8000)	Y amorph	Cl-water = gΔy Wh by NH_4OH
Nicotine	colorl	colorl	colorl—Y—R	colorl—Y—rWh	colorl	Y	colorl	Wh (1 : 2500)	PΔdt Wh (1 : 40,000)	r Br (1 : 250,000)	Y only in conc soln	

*Tables reproduced from The Merck Index, Fourth Edition, by permission of Merck & Co., Inc.

TABLE 227 (CONT'D)

Reactions of the More Important Alkaloids and Glucosides—*Continued*

	COLOR REACTIONS							PRECIPITATES				Various Other Reactions
	Conc. H_2SO_4	Conc. HNO_3	H_2SO_4 + HNO_3 (Erdmann)	H_2SO_4 + Ammon. (or Sod.) Molybdate 100 : 1–5 (Fröhde)	H_2SO_4 + Ammon. meta Vanadate 200 : 1 (Mandelin)	H_2SO_4 + Selenous Acid 200 : 1 (Mecke)	H_2SO_4 + Formaldehyde 25 : 1 (Marquis)	$HgI_2 . 2KI$	$BiI_3 . 4KI$	I . KI	Picric Acid	
Papaverine	colorl–pale P ΔdkV by heat	y–dk R	gBlΔdk R	GΔBlΔVΔch R by heat	bl G–Bl	gΔdk BlΔV by heat	RΔOr	Wh (1 : 1000)	Or R (1 : 10,000)	dk R (1 : 50,000)	Y (1 : 500)	Cl-water = g Δ Br by NH_4OH
Physostigmine	colorl–Y	YΔol GΔR	YΔR		gYΔyBr by heat	br YΔlt br R by heat	colorl	Wh (1 : 5000)	ppt	r Br (1 : 25,000)	only in conc soln	Alkali hydroxides = intense RΔ YΔGΔBl
Pilocarpine	colorl		colorl	colorl	YΔlt GΔBl	colorl	colorl–yΔR		ppt	ppt	ppt	$H_2SO_4 + K_2Cr_2O_7$ = G color
Piperine	blood RΔbr Bk	OrΔblood R by heat	rBrΔdk gBk by heat	RΔalm BkΔdkG	blood RΔBk	gBr	R	yWh				
Quinidine	colorl	colorl	colorl	g	bl G		slt G	1 : 5000	brick RΔdt Wh	Br	ppt	Cl-water + NH_4OH = as under Quinine $K_4Fe(CN)_6$ = y Wh ppt $K_2Cr_2O_7$ = Y ppt
Quinine	colorl–lt yΔ Br by heat	colorl	colorl	colorl	colorl	colorlΔlt Br by heat	colorl	Wh (1 : 125,000)	Or R (1 : 50,000)	r Br	amorph (1 : 40,000)	Cl-water + NH_4OH = G flocks sol in exs (emerald G soln); soln neutralized = Bl–VΔG by NH_4OH $K_2Cr_2O_7$ = Y ppt Periodic acid liberates free I
Solanine	BrΔrV	colorl	rYΔdt R	OrΔrBrΔY	OrΔRΔV	rYΔgrayish Br by heat	Br	none	Y	none in acid soln	ppt in conc soln	
Sparteine	colorl	colorl	colorl		colorl						Y	
Strophanthin	lt YΔGΔBr	lt BrΔa series of colors by heat						none	none	none	none	$H_2SO_4 + K_2Cr_2O_7$ = gBr HCl = YΔa series of colors by heat HIO_4 = lt Br $FeCl_3$ = gY color or slt ppt
Strychnine	colorlΔOr Y by KOH	colorlΔY by heat	colorl	colorl	VBlΔR	colorl	colorlΔgBr by heat	wh (1 : 15,000)	lt Y (1 : 250,000)	rBr (1 : 50,000)	Y (1 : 20,000)	$H_2SO_4 + K_2Cr_2O_7$ = VBlΔRΔG HIO_4 = wine R
Theobromine	colorl	colorl	colorl	colorl	colorl			none		ppt in conc soln	none	Evap. w. Cl-water or HNO_3 & add NH_4OH = P color (murexid test)
Thebaine	blood RΔyR	Y	R	Or				ppt (1 : 5000)	Or (1 : 10,000)	ppt (1 : 5000)	Y	
Veratrine	YΔVΔR by heat	Y	Y–OrΔchR	YΔOrΔch R	YΔch RΔP	lm YΔol GΔ br V by heat	yBrΔrBr by heat	yWh in conc. soln.	ltYΔcanary Y in conc soln	Br (1 : 15,000)	1 : 10,000	$H_2SO_4 + K_2Cr_2O_7$ = rBr H_2SO_4 + sugar = grad. deep GΔBl HCl = deep VΔintense R by heat
Yohimbine	colorlΔdt G by $K_2Cr_2O_7$	colorlΔintense YΔOr R by heat	bl BkΔg–yBr		Bl	InBl	dt GΔbr V by heat	Wh			y	

Cork in a containing flask, shake, and set aside for from twelve to eighteen hours, agitating from time to time.

Filter the mixture, using a filter pump, and a Büchner funnel.

Wash the residue with successive small portions of alcohol of the same strength and acidulation as the filtrate.

Evaporate off all the alcohol in a water-bath.

Add water just perceptibly acidulated with sulphuric acid in quantity barely enough to bring the soluble matter into limpid solution.

Stir the whole thoroughly.

Filter out the insoluble matter.

The filtrate must be clear.

Shake out the filtrate with the immiscible solvents as follows:

(1) While acid, with chloroform in several small successive portions;
(2) On making the liquid alkaline with ammonia, with four small portions of a mixture of chloroform and ether;
(3) After slight acidulation and warming, with hot amyl alcohol in several portions;
(4) After making alkaline with ammonia and warming with hot amyl alcohol as the solvent for morphine, this being applied in repeated portions.

The immiscible solutions, (1), (2), and (3), being respectively chloroform, chloroform-ether, and amyl alcohol, should be once or twice shaken out with a small quantity of acidulated water.

These small water-washings should be added to the aqueous liquid treated in (4).

The final morphine extraction in (4) is made with the liquid and solvent both hot, and continued in at least three successive portions, and until, on evaporating 1 or 2 c.c., a negative result is obtained with one of the more delicate tests.

Filter the united amyl alcohol solutions (first wet the filter paper with amyl alcohol).

Cool.

Shake out with water in very small portions until the resulting washings give no precipitate with barium chloride.

Measure the amyl alcohol solution so washed. Hold in reserve for qualitative and quantitative tests.

To identify morphine positively, we must find the crystals.

To obtain these crystals, use the final residue, mix with warm alcohol, drop by drop, and then watch microscopically. Morphine crystallizes in single and group columns of orthorhombic system with hexagonal surface.

(B) Qualitative Tests

The Marquis Test

Reagent.—

3 c.c. of concentrated sulphuric acid.
2 drops of 40% formaldehyde (Formalin).

Technic.—

Obtain a dry residue by evaporation of some of the amyl alcohol filtrate. This should be on white porcelain.

Touch with the above reagent from the point of a glass rod.

An intense purple red color, changing to violet, and finally to blue is observed if morphine is present.

A marked reaction is obtained with 0.02 mg. morphine.

Codeine and apomorphine do not give the initial purple, but produce a violet changing to blue.

Oxydimorphine shows a green color, which changes to a flame red.

Dionine gives a dark blue violet.

Heroine shows the same colorations as does morphine.

Deniges' Test

Place in a test tube 10 c.c. of dilute solution of the residue. (Not less than 0.03 gram per liter.)

Add 1 c.c. hydrogen peroxide

and 1 c.c. ammonium hydroxide solution.

Add 1 drop of a 4% solution of copper sulphate.

Shake.

At once a rose red to a deep red color appears, depending on the concentration of the morphine present.

Codeine, thebaine, papaverine, narceine, and narcotine give negative results.

Oxydimorphine, apomorphine, heroine, and dionine give somewhat similar results.

(C) Quantitative Tests

Evaporate the amyl alcohol solution to dryness.

Dissolve this dried residue in a known amount of N/10 sulphuric acid solution.

Titrate the excess of acid with N/50 potassium or sodium hydroxide solution, using cochineal as indicator.

Each c.c. of N/10 sulphuric acid consumed in the combination with morphine in the residue corresponds to 0.028516 gram of anhydrous morphine.

Example:

15 c.c. N/10 sulphuric acid were used to dissolve the residue.

40 c.c. N/50 sodium hydroxide were used in the titration. This corresponds to 8 c.c. of N/10 NaOH.

8 c.c. of N/10 sulphuric acid were present, therefore, in excess.

15–8 equals 7 c.c. of N/10 sulphuric acid which combined with the morphine.

7 × 0.028516 equals 0.199612 gram of morphine present.

0.13 gram, or 2 grains, of morphine sulphate may be poisonous when swallowed and undoubtedly endanger the life of a person unaccustomed to the drug.

The average **minimum fatal dose of morphine** for an adult is about 1 grain, according to Haines.

THE APPLICATION OF ULTRAVIOLET ABSORPTION SPECTROSCOPY IN ANALYTICAL TOXICOLOGY*

Ultraviolet absorption spectra have been used for many years to establish the identity and structure of organic as well as inorganic compounds. The Beckman photoelectric spectrophotometer with ultraviolet attachments provides an instrument that is convenient, easily manipulated, and accurate so that ultraviolet absorption methods can be adapted for routine laboratory examinations. In the last ten years, numerous procedures have been developed for the identification and determination of a large number of compounds, many of which are of special interest to the toxicologist. These include sedatives, analgesics, antihistaminics, alkaloids, insecticides, and other aromatic poisons. Ultraviolet methods have the added advantage of retaining the sample for other tests. They are rapid and provide a highly specific identification and quantitative estimation for low concentrations of substances; thus the toxicologist can use ultraviolet analysis as a routine, rapid screening procedure for many compounds in all cases of accidental or unexplained deaths. For example, since the widespread use of barbiturates, it is necessary to rule out these hypnotics as a contributory cause of death in auto accidents, home accidents, etc. Analysis of a small sample of blood by the ultraviolet procedure detects barbiturates in concentrations well below the toxic range.

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*After Kaye and Goldbaum in Gradwohl: Legal Medicine, The C. V. Mosby Co., 1954.

The basic laws governing the absorption of radiant energy are those of Beer-Bouguer (page 210).

Spectral Analysis.—The identification of compounds by their spectra is made by finding the curve of a known compound that agrees with the unknown. In spite of their empirical nature, spectrophotometric identifications are widely accepted, provided the work is done carefully. When the absorption spectrum of a compound is determined, identification is attempted first from the curve shape. Of primary concern is what maxima and minima occur with respect to wave length and then what are their relative heights. This is at best an identification of a particular chromophore group. However, even if two compounds have a similar chromophore group, they usually do not have identical spectra over the entire range of the ultraviolet as illustrated by the substituted barbiturate derivatives, Table 229. Generally, if compounds have many points of inflection which agree, identification is more certain than if there are relatively few. A compound like quinine is relatively easy to identify because it has a number of inflection points, while other compounds such as strychnine with only one maximum are not conclusively identified from their spectra.

It is important not to fail in a tentative identification of a compound because of trivial differences from small amounts of impurities. As a rule, impurities are more likely to cause greater changes at the lower wave lengths. An important principle in ultraviolet spectrophotometry is that, in almost all instances when two or more substances are present together, their absorbencies are additive. Thus the absence of absorbence at definite wave lengths precludes the presence of many substances.

The external solution conditions alter the absorption spectra of many compounds. This is important because, first, conditions must be kept constant to avoid errors in qualitative and quantitative analysis, and, second, changing the solution conditions may provide a means for more specific identification of compounds. Such factors as solvent, temperature, pH may increase, decrease, or completely change the absorption spectra of a compound. Any one or combination of these conditions may be used to further aid the identity of a substance.

As a general statement, the transmittancy range of 10 to 50 per cent is desirable in accurate work. High transmittancy should be avoided because inaccuracies arise due to accidental dirt on cuvettes, effects of slight turbidity in both the unknown and in the reference cell. Similarly, too low transmittancy should be avoided due to inaccuracies of the readings and the failure to follow Beer's law in some instances. The sensitivity of a procedure may be increased by resorting to cuvettes with longer light paths or by using 1 cm. light path microcuvettes with a capacity of 0.5 c.c.

Ultraviolet Spectrophotometric Analysis of Drugs.—The toxicologist is continually confronted with specimens of medicines, pills, capsules, and other substances submitted for analysis. These types of samples generally require no separation procedures. The substances are present in relatively high concentrations requiring usually considerable dilution with acid or base to arrive at the proper absorbency. At these high dilutions, the interference from absorption of preservatives and fillers usually can be eliminated. When two absorbing substances are present, it may be possible to determine both if one component can be identified by its absorption at the higher wave lengths. Then its absorption band can be subtracted from the total absorbencies and the remaining substance in turn identified by its characteristic absorption band. When two known components are present, the concentration of both can be determined by setting up a simultaneous equation and solving at two wave lengths. This procedure is used in the determination of aspirin, Phenacetin, and caffeine in pills.

Ultraviolet Spectrophotometric Analysis of Biological Samples

The isolation of relatively small amounts of organic drugs from normally occurring absorbing substances in blood, urine, and tissues presents a problem in the ultraviolet spectrophotometric analysis of biological samples. In general, the isolation procedure consists of adjusting the biological sample to the proper pH and then extracting with an organic solvent. The organic drug is transferred to the water phase by extracting the solvent with an acid or alkaline solution to form a water-soluble salt. The absorbencies of the water solution are determined in an ultraviolet spectrophotometer.

The solvents that have been used in the extraction of biological samples in order of increasing polarity are petroleum ether, hexane, carbon tetrachloride, chloroform, ethylene dichloride, ether, and the higher molecular weight alcohols. The choice of solvent depends upon the distribution coefficient of the particular component in water and in solvent. Most of the organic compounds encountered in toxicology require the use of somewhat polar solvents. Chloroform appears to be the solvent of choice for almost all drugs. Ethylene dichloride is the best polar solvent for extracting nervous tissue. Unlike chloroform, no emulsions occur.

The presence of absorbing substances in the final extract interferes with the specificity, sensitivity, and accuracy of the determination. To reduce absorbing substances, solvents are distilled, run through silica gel, or washed with acids and alkali. To offset any absorption by the reagents, a water sample is carried through the same extraction process as the unknown and used as the reference solution.

The use of the least polar solvent possible will reduce the extraction of normally absorbing substances present in biological materials. Adjusting the sample to a specific pH before extraction with solvent or washing the solvent layer with a solution of suitable pH after extraction of the sample also will reduce the extraction of interfering substances. For example, in the determination of barbiturates, strongly acid substances are not extracted by the chloroform when the biological sample is adjusted to pH 7.4. In the determination of quinine, the solvent extract of a biological sample is washed with an alkaline solution of pH 9.0 to reduce interfering absorbing substances.

When a drug has a high absorbency index or is present in relatively high concentrations as in acute poisonings, interference from normally absorbing substances may be insignificant. Pentothal, a thiobarbiturate, is an example of a compound having a high absorbency index. This drug is readily determined by ultraviolet spectrophotometric analysis.

A correction for the absorbency of the impurities at the wave length where the component is to be determined can be made if a straight line exists when the absorbencies of the impurities are plotted against wave lengths. This procedure has been used in the determination of barbiturates, caffeine, and benzene hexachloride.

In some instances, interfering substances can be eliminated if the absorbencies of the component change with pH while the absorbencies of the interfering substances remain the same. Another procedure used in overcoming interference from impurities is to bring a change in absorbency of the component by chemical means. In the determination of antipyrine, the absorbency of the solution is determined at 350 mμ, then sodium nitrite is added. If there is an increase in the absorbency at 350 mμ, 4-nitroso-antipyrine has been formed, indicating the presence of antipyrine. The change in absorbency is related to the concentration of antipyrine in solution.

TABLE 228.—COMPOUNDS ISOLATED BY STEAM DISTILLATION

WAVE LENGTH Mμ	SPECTRA IN DISTILLED WATER				SPECTRA IN HEXANE			
	MAXIMA	$E^{1\%*}_{1\text{ cm.}}$	MINIMA	$E^{1\%}_{1\text{ cm.}}$	MAXIMA	$E^{1\%}_{1\text{ cm.}}$	MINIMA	$E^{1\text{ cm.}}_{1\%}$
330	β-Naphthol	95						
303	Methyl salicylate	340						
295	α-Naphthol	350	β-Naphthol	45				
290					Camphor	5		
285	β-Naphthol	185						
280	Aniline	165	β-Naphthol	170				
	Camphor	3						
274	β-Naphthol	250						
	Thymol	131						
273	Lysol and cresol	142.5						
270	Phenol	147						
	Nitrobenzene	780						
267							Camphor	2.6
262			Methyl salicylate	30				
260			Lysol and cresol	107.5				
			Aniline	75				
255	Benzene	6.5			Benzene	7.5		
250			α-Naphthol	90				
248			β-Naphthol	120				
245			Thymol	11	Camphor	6		
238	Methyl salicylate	820	Phenol	10				
235			Camphor	7				
230	Aniline	920						
228			Nitrobenzene	240				

*$E^{1\%}_{1\text{ cm.}}$ = extinction coefficient.

Purification Procedures Used in the Preparation of Samples for Ultraviolet Spectrophotometry

The residues of the acid and basic group extracts in the Stas-Otto procedure (pages 2285, 2302, and 2313 ff.) contain large amounts of impurities that interfere with the identification of organic drugs by ultraviolet spectrophotometry. The following technics have been successfully applied to the purification of many organic drugs:

The procedure of microvacuum sublimation of Gettler, Umberger, and Goldbaum is an effective means of purifying a large number of drugs such as barbiturates, salicylates, alkaloids, and other compounds. Micro amounts of the sublimate can be easily isolated, dissolved in a suitable solvent, and its ultraviolet absorption spectrum determined.

Column absorption chromatography has been used to separate and purify barbiturates and alkaloids. The technic of ion exchange of resins also has been applied to the determination of narcotics. In both these processes, it is possible to obtain relatively pure compounds for ultraviolet spectrophotometric analysis. Paper iontophoresis and paper chromatography furnish an excellent purification procedure for the separation and final determination of alkaloids by ultraviolet spectrophotometry. The application of these technics is illustrated on page 2318 in the determination of morphine in urine.

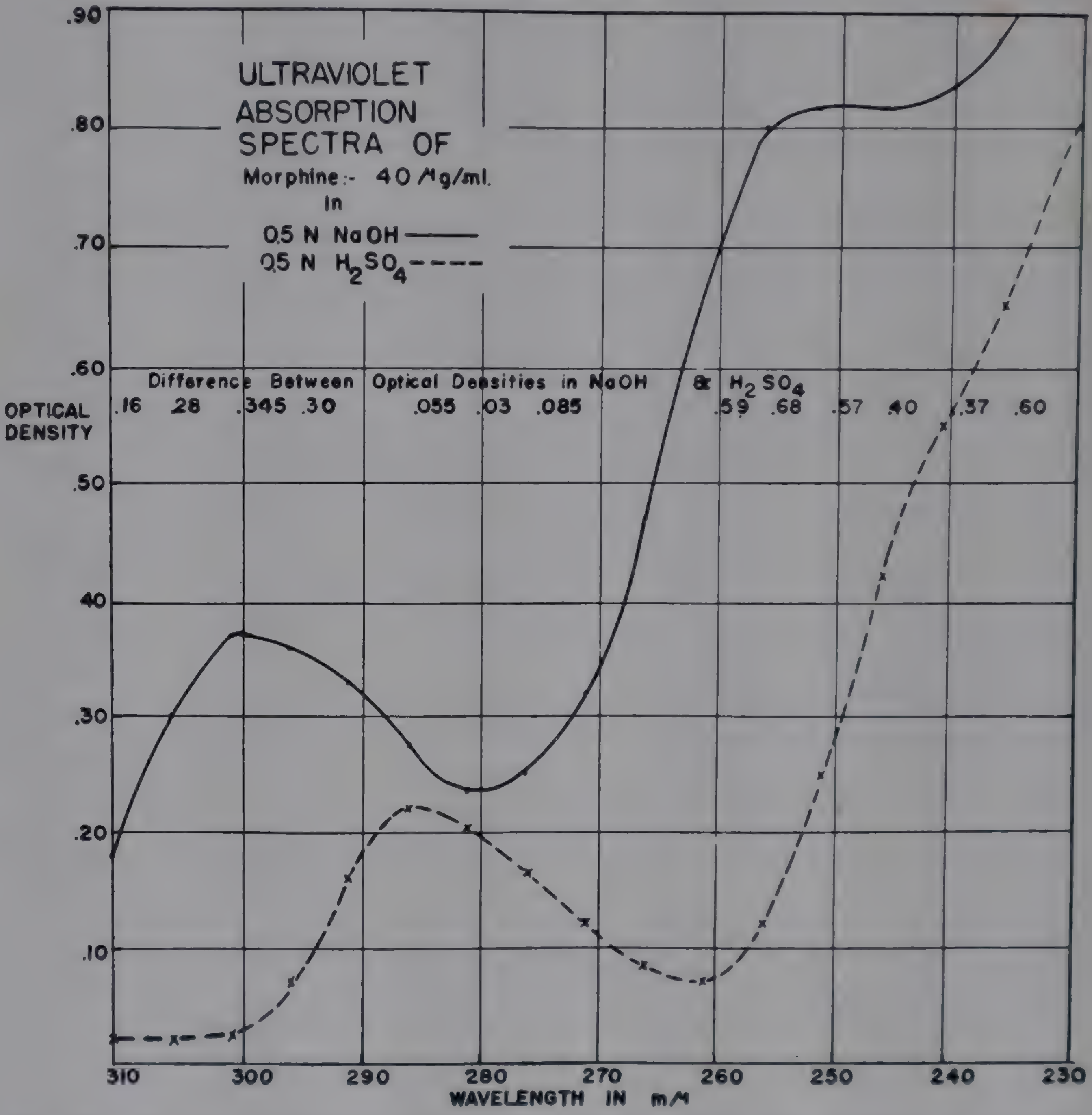

Fig. 703.—Ultraviolet absorption spectrum of morphine. (Kaye and Goldbaum in Gradwohl: Legal Medicine, The C. V. Mosby Co., 1954.

Ultraviolet Spectrophotometric Examination of Organic Compounds Appearing in the Stas-Otto Group Separations

Volatile Distillates.—Many aromatic compounds that are volatile with steam can be readily identified and quantitatively estimated by their characteristic ultraviolet absorption spectra. The absorbencies of the steam distillate of either stomach contents or tissue are determined at various wave lengths from 330 to 225 mμ, using distilled water as a reference solution. Insignificant absorbencies at specific wave lengths can preclude the presence of many compounds. Table 228 shows the maxima and the minima and the $E^{1\%}_{cm}$ for some compounds that may appear in the steam distillate. Since the $E^{1\%}_{cm}$ of benzene and camphor are low, a tenfold increase in sensitivity can be made by extracting 50 c.c. of the steam

TABLE 229.—COMPOUNDS ISOLATED IN THE ACID-SOLVENT GROUP

WAVE LENGTH Mμ	SPECTRA IN 0.5 N NaOH				SPECTRA IN BORATE BUFFER pH 10.5		SPECTRA IN 0.5 N HCl			
	MAXIMA	$E_{1\,cm.}^{1\%}$	MINIMA	$E_{1\,cm.}^{1\%}$	MAXIMA	$E_{1\,cm.}^{1\%}$	MAXIMA	$E_{1\,cm.}^{1\%}$	MINIMA	$E_{1\,cm.}^{1\%}$
315	Dicumarol	790								
305	Thiobarbital	1100								
	Pentothal	1100								
300	Salicylic acid	260					Salicylic acid	275		
290							Pentothal	960		
285							Thiobarbital	1000		
280	Acetanilid	110								
275	Theophylline	650								
273	Caffeine						Theophylline (pH 6.4)	555		
270			Salicylic acid	60						
265									Salicylic acid	450
260			Acetanilid	47.5						
			Dicumarol	140						
255	5-5, Substituted barbituric acid derivatives	290–310							Pentothal	150
									Thiobarbital	200
250							Picrotoxin	22.5		
245	n-Methyl, barbituric acid	250	Caffeine				Phenacetin	670		
			Theophylline	130						
	Phenacetin	720	Thiobarbital	130						
			Pentothal	70						
243										
242							Acetanilid	40		
240	Picrotoxin	107.5			5-5, Substituted barbituric acid derivatives	435			Theophylline (pH 6.4)	120
235			5-5, Substituted barbituric acid derivatives	170–230			Salicylic acid	640		
230	Acetanilid	555	Phenacetin	635						

TABLE 230.—COMPOUNDS ISOLATED IN THE BASIC-SOLVENT GROUP

WAVE LENGTH Mμ	SPECTRA IN 0.5 N NaOH				SPECTRA IN 0.5 N HCl			
	MAXIMA	$E^{1\%}_{1\text{ cm.}}$	MINIMA	$E^{1\%}_{1\text{ cm.}}$	MAXIMA	$E^{1\%}_{1\text{ cm.}}$	MINIMA	$E^{1\%}_{1\text{ cm.}}$
350					Quinine and quinidine	172.5		
344					Chloroquin	570		
337							Chloroquin	470
330	Chloroquin	360			Chloroquin	525		
	Quinine and quinidine	95						
325	Nupercaine	130					Quinine and quinidine	132.5
320					Quinine and quinidine	145		
					Stilbamidine	190		
					Nupercaine	270		
317					Cinchonidine	255		
315	Stilbamidine	270			Narcotine	95		
314	Pyribenzamine	192.5			Pyribenzamine	340		
					Methapyrilene	270		
313					Tetracaine	800		
305	Brucine	235	Quinine and quinidine	87.5				
304					Methapyrilene (pH 6.3)	157		
300	Morphine	92.5			Brucine	220		
	Heroin	68.8						
295			Nupercaine	85	Methadone	20		
291					Hydrastine			
290	Dilaudid	82.5						
	Cinchonidine	170						
285	Dionin	50	Brucine	152.5	Morphine	55	Brucine	165
	Codeine	53.8			Codeine	58.8	Chlorequin	65
	Quinine and quinidine	72.5			Dionin	60		
280	Narcotine	86.3	Chloroquin	75	Heroin	46.3		
	Butacaine	410	Heroin	45	Dilaudid	41.3		
			Morphine	58.8	Emetine	116.3		
278					Epinephrine	230		
275			Pyribenzamine	47.5			Methadone	14
			Dilaudid	63.8				
273					Theobromine (pH 5.7)	550		
					Caffeine (pH 3.0)	510		
							Nupercaine	42.5
270					Quinine and quinidine	27.5	Narcotine	100
					Butacaine	32.5	Stilbamidine	
					Procaine	55	Methapyrilene	80
265	Brucine	320	Codeine	23.8	Pentamidine	615	Hydrastine	
	Procaine	630	Dionin	22.5	Brucine	335	Dilaudid	25
							Cinchonidine	25
							Pyribenzamine	40
260	Atropine	10.4	Narcotine	40	Methadone	19.5	Procaine	45
	Dexedrine	15.2	Quinine and quinidine	84.5			Morphine	17.5
	Benzedrine	16					Dionin	27.5
							Codeine	26.3
W257	Chloroquin	520			Demerol	8.8		
					Chloroquin	490		
255	Strychnine	285			Ephedrine	8.8	Methadone	18
					Strychnine	295	Butacaine	26.5
							Heroin	17.55
							Emetine	25
252					Dexedrine	12		

Table 230.—Cont'd

WAVE LENGTH Mμ	SPECTRA IN 0.5 N NaOH				SPECTRA IN 0.5 N HCl			
	MAXIMA	$E^{1\%}_{1\,cm.}$	MINIMA	$E^{1\%}_{1\,cm.}$	MAXIMA	$E^{1\%}_{1\,cm.}$	MINIMA	$E^{1\%}_{1\,cm.}$
					Benzedrine	13		
250	Pentamidine	590	Cinchonidine	70	Quinine and quinidine	587.5	Epinephrine	80
	Pyribenzamine	635					Demerol	6.4
							Tetracaine	50
247					Nupercaine	715		
245			Chloroquin	455			Chloroquin	440
							Theobromine (pH 5.7)	126
							Caffeine	152
240	Butacaine	90	Atropine	7.9	Pyribenzamine	570		
			Brucine	140				
238					Methapyrilene	610		
235	Stilbamidine	295			Chloroquin	580	Brucine	115
					Cinchonidine	1220	Cocaine	480
232			Dexedrine	6.2			Dexedrine	4.4
			Benzedrine	7.2			Benzedrine	6
231							Nupercaine	270
230	Quinine and quinidine	980	Strychnine	132.5	Procaine	550	Chloroquin	570
			Procaine	125	Stilbamidine	225	Quinine and quinidine	347.5
							Strychnine	97.5
							Pentamidine	160
228	Cinchonidine	1190						

distillate with 5 c.c. of n-hexane. The absorbencies of the hexane extract which will quantitatively remove benzene and camphor can be determined. The reference solution is n-hexane previously shaken with 50 c.c. of distilled water.

Acid Solvent Extract.—The examination of this group of compounds by ultraviolet spectrophotometry is best made after a purification procedure such as vacuum sublimation. The purified compound is dissolved in an appropriate solvent and its ultraviolet spectrum determined. No purification is necessary when large amounts of compounds are present in the residue. For many drugs in the group, a direct extraction of small samples (1 to 5 gm.) is made for their determination by ultraviolet spectrophotometry (Fig. 703). Table 229 lists the maxima and minima $E^{1\%}_{cm}$ of many drugs in this group.

Alkaline Solvent Extracts.—Purification of the extracts is generally required before examination by ultraviolet spectrophotometry. Vacuum sublimation, chromatography, paper iontophoresis are effective purification procedures. The maxima and minima $E^{1\%}_{cm}$ of basic compounds are shown in Table 230.

Paper Iontophoresis and Paper Chromatography in the Determination of Alkaloids*†

The moving of mixtures on filter paper strips is a valuable microanalytical method for purification and identification of alkaloids. In paper iontophoresis, the mixture to be separated is applied at some intermediate position on a filter paper strip. An electropotential is applied across the ends of the filter paper strips saturated with an electrolyte. Separation of the constituents depends upon the fact that certain charged substances exhibit relative differences in mobilities at different pH values under the influence of an electrical field.

A suitable apparatus consists of a Lucite box with a center partition dividing it into anode and cathode compartments. Platinum foil electrodes dip into the electrolyte solution in the anode and cathode sides. The two com-

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*Kaye, Sidney, in Gradwohl: Legal Medicine, St. Louis, 1954. The C. V. Mosby Co.
†See page 221 for paper electrophoresis.

partments are connected by a piece of Tygon tubing to permit initial equilibration of the liquid level of anolyte and catholyte in order to prevent electrolyte flow currents due to "siphoning." During operation, this tubing is clamped shut. Filter paper strips are supported by a glass support frame with their ends dipping into the electrolyte compartments. A peaked roof of the cover of the cell permits any electrolyte condensing on the cover to drain back into the electrolyte compartments without dripping upon the filter paper. For the movement of alkaloids, the electrolyte is 1N acetic acid. The filter paper strips can be Whatman No. 1, 2, 3, or 50. Three hundred to 600 volts are applied for ½ to 2 hours.

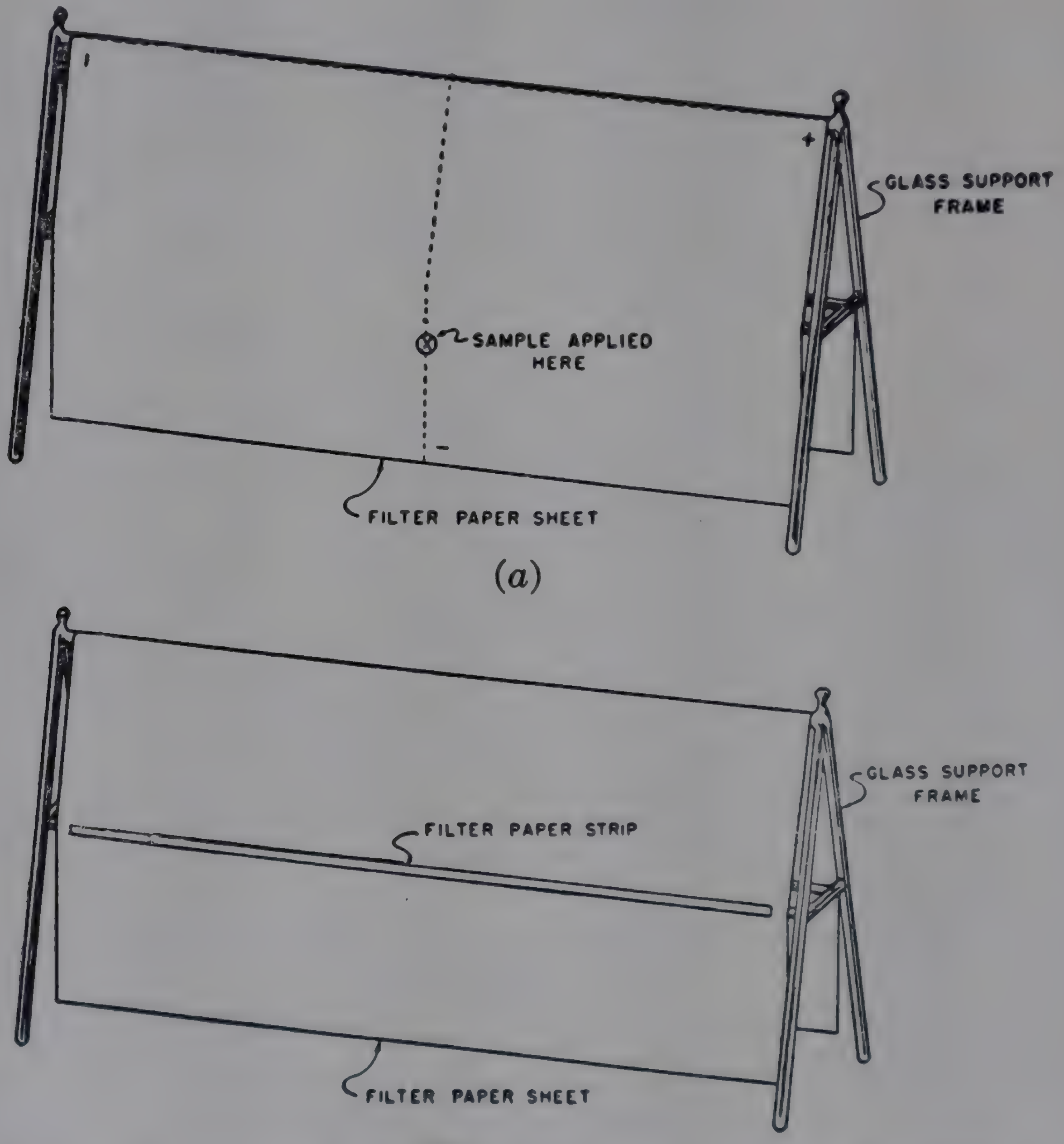

Fig. 704.—Durrum's apparatus for electrophoresis and iontophoresis. (From Gradwohl: Legal Medicine, The C. V. Mosby Co.)

In paper chromatography, the movement of a mixture along a strip of filter paper is accomplished by means of a solvent front. The ratio between the distance traversed by a component and the distance traveled by the solvent is known as the *Rf value*. This value depends mainly on the distribution coefficient of a compound in water and an immiscible solvent. It provides an identifying characteristic for a given compound. The mixture to be separated is applied near the end of a strip of filter paper which is dipped into the mobile solvent. The solvent front moves up or down the paper, depending on whether an ascending or descending technic is used. The chromatography apparatus consists of an enclosed tank or glass jar with a rack to suspend the filter paper strips and a container to hold the mobile solvent. The atmosphere inside the tank is saturated with water and the immiscible solvent. In the separation of alkaloids, Munier and Macheboeuf[1] use a mixture of n-butanol, glacial acetic acid, and water for the mobile solvent, while Carless and Woodhead[2] use filter paper strips saturated with N/15 phosphate buffer and a mobile solvent of n-butanol saturated with water. Whatman No. 1, 2, 3, or 50 filter paper strips can be used. The time necessary for adequate movement of the solvent front is from 6 to 15 hours.

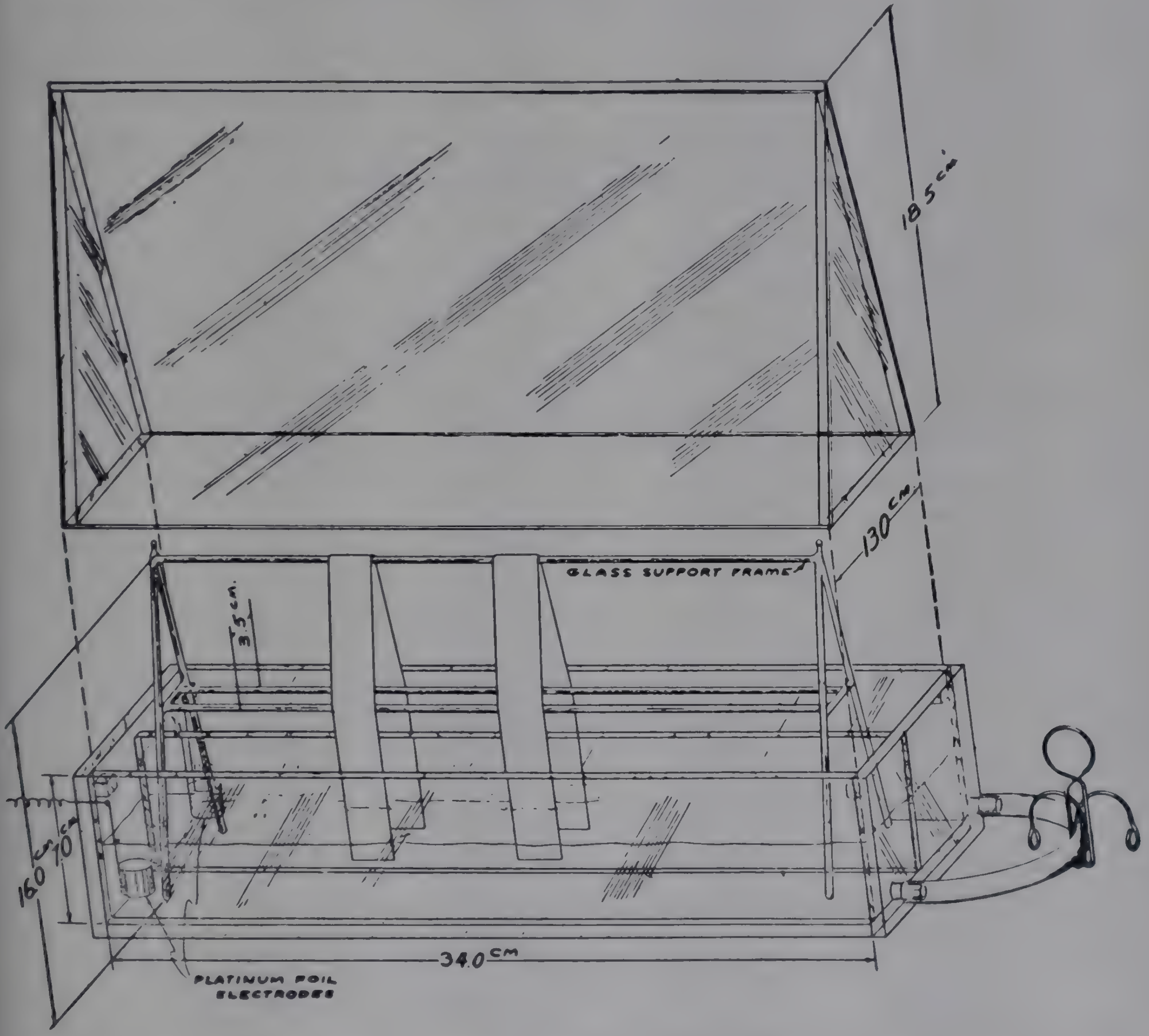

Fig. 705.—Durrum's apparatus for electrophoresis and iontophoresis. (From Gradwohl: Legal Medicine, The C. V. Mosby Co.)

Detection of Alkaloids

After separation of a mixture by paper iontophoresis or paper chromatography, dry the filter paper strips and examine for the presence of alkaloids.

Under ultraviolet light, the presence of fluorescent alkaloids like quinine, quinidine, Atabrine, and Nupercaine are readily discernible.

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[1] Bull. Soc. Chem. Biol. 32: 192, 1950.

[2] Nature 168: 203, 1951.

Spraying the paper with such alkaloidal reagents as potassium iodobismuthate[3] and potassium iodoplatinate[3] will form orange and black complexes, respectively, with many alkaloids.

Recovery of the alkaloid is possible by cutting out the colored complex and decomposing it with an alkaline solution containing sodium sulphite.

The alkaloid can be isolated by extraction with a suitable solvent.

When a hard acid- and base-washed filter paper such as Whatman No. 50 is used in paper iontophoresis or paper chromatography, it is possible to apply directly on the paper any of the color-producing alkaloidal reagents containing concentrated sulphuric acid. Such alkaloidal reagents as Meckes, Mandelin, Marquis (see Bamford's systematic scheme, page 2322), and others can be applied directly to the dried filter paper, producing their characteristic colors with many alkaloids.

The use of paper iontophoresis and paper chromatography in the determination of morphine illustrates this microanalytical technic.

Morphine in Urine, Golbaum[4] and Kazyak[5]

Reagents.—

Concentrated Hydrochloric Acid.—

Sodium Carbonate, Solid.—

Solvent.—

Isobutyl alcohol	2 parts
Chloroform	8 parts

Half-Saturated Sodium Borate Solution.—

Adjust to pH 9.0.

0.5N Sodium Hydroxide Solution.—

95% Alcohol.—

Whatman No. 1 Filter Paper.—

Whatman No. 50 Filter Paper.—

1N Acetic Acid.—

Iodoplatinate Reagent.[3]**—**

Combine 5 c.c. of 5% platinum chloride in N/1 hydrochloric acid
45 c.c. of 10% potassium iodide
100 c.c. of distilled water.

Store in a dark glass-stoppered bottle.

Mobile Solvent of Munier and Macheboeuf.—

This is a mixture of 100 c.c. of n-butanol and 25 c.c. of glacial acetic acid, with enough water added to the mixture to produce a slight turbidity.

Mobile Solvent of Carless and Woodhead.—

This is n-butanol saturated with M/15 phosphate buffer pH 5.0.

For Quantitative Determinations of Morphine.—

2% Sodium Sulphite Solution in Half-Saturated Sodium Borate pH 9.0.—

0.1N Sodium Acetate-Acetic Acid Buffer pH 4.0.—

Technic.—

(A) **Preparation of the Specimen.—**

Reflux a sample of urine for 3 hours with 1/10 its volume of concentrated hydrochloric acid to completely hydrolyze any combined morphine.

Adjust the reaction of 50 c.c. of the urine with solid sodium carbonate to pH 8.5 ± 0.5.

Extract with 250 c.c. of isobutyl alcohol-chloroform solvent.

Separate the solvent layer.

Shake with 25 c.c. of half-saturated sodium borate solution pH 9.0 to remove some interfering substances.

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[3] Zaffaroni, A., Burton, R. B., and Keutmann, E. H.: J. Biol. Chem. 177: 109, 1949.

[4, 5] In press.

Run the solvent layer through filter paper.

Determine the volume of the aliquot (aliquot 1).

Separate the basic constituents by shaking the solvent with 25 c.c. of 0.5N sodium hydroxide.

Discard the solvent layer.

Acidify the alkaline layer containing morphine as the sodium salt, using first concentrated phosphoric acid, then adjusting the reaction to pH 8.5 ± 0.5 by adding solid sodium carbonate.

Extract with 125 c.c. of solvent.

Run the solvent layer through filter paper to remove any traces of water.

Record the aliquot (aliquot 2).

Evaporate to dryness in a beaker containing 2 c.c. of 1N hydrochloric acid.

(B) **Purification of Residue: Paper Iontophoresis.**—

Take up the residue in 1.5 c.c. of 95% ethyl alcohol.

Streak 1 c.c. across the center of a strip of Whatman No. 1 filter paper 13 cm. wide and 32 cm. long.

Evaporate the alcohol. This can be speeded by applying a stream of hot air from an electric hair dryer.

Evaporate 0.1 c.c. of the alcohol in the center of a filter paper strip 5 cm. wide and 31 cm. long.

Streak the remainder of the alcohol across a strip of Whatman No. 50 filter paper.

Suspend the three filter strips in an electrophoresis or iontophoresis apparatus and wet with the electrolyte 1N acetic acid.

Apply an electrode potential of 600 volts across the ends of the filter paper strips.

After 1 hour, remove the strips, dry, and test for alkaloids.

(C) **Test for Alkaloids.**—

Spray the wide and narrow strips of Whatman No. 1 filter paper prepared as above with iodoplatinate reagent.

The presence of morphine is indicated by the appearance of a black iodoplatinate-morphine complex. An estimation of the morphine concentration can be made by comparing the black spot on the narrow strip with those produced by known morphine concentrations.

Color tests for morphine can be made by applying Marquis, Meckes, and other reagents directly on the Whatman No. 50 filter paper. These reagents are capable of producing characteristic colors with less than 5 micrograms of morphine.

(D) **Paper Chromatography.**—

As described under paper iontophoresis, aliquots of the alcoholic residue can be streaked across the base of strips of Whatman No. 1 and No. 50 filter paper and suspended in a chromatographic apparatus.

With the method of Munier and Macheboeuf their solvent is used.

After the solvent front moves from 20 to 30 cm., dry the filter paper strips.

Continue with the tests for detection and identification of morphine as above.

The Rf value for morphine is approximately 0.60.

If the procedure of Carless and Woodhead is used, saturate the filter strips with M/15 phosphate buffer pH 5.0.

The mobile solvent is described above.

The Rf value for morphine is approximately 0.17.

A quantitative determination and specific identification of morphine can be made using the wide strip of Whatman No. 1 filter paper.

Cut out the section of paper containing the black iodoplatinate-morphine complex and transfer to a glass-stoppered cylinder containing 2 c.c. of a 2% sodium sulphite solution in half-saturated sodium borate pH 9.0, which destroys the complex and liberates free morphine.

Add 25 c.c. of solvent and shake the cylinder.

Remove the water layer.

Filter the solvent layer into another glass-stoppered graduated cylinder.

Measure the aliquot (aliquot 3).

Add 4 c.c. of 0.1N sodium acetate-acetic acid buffer pH 4.0, and shake.

The buffer solution now contains morphine as the acetate.

Transfer to a cuvette of a Beckman ultraviolet spectrophotometer.

Determine absorbencies at a number of wave lengths between 310 and 245 mμ, using as a reference solution a sodium-acetate-acetic acid buffer extract of 25 c.c. of the solvent.

(E) **Results.**—

To compare the absorbencies obtained at pH 4.0 with those on the alkaline side, pipette 2 c.c. of the test solution and of the reference solution into tubes containing 1 c.c. of 1.5N sodium hydroxide.

Return the alkaline solutions to their respective cuvettes, and determine absorbencies at the previous wave lengths, correcting for dilution with alkali by multiplying by 1.5.

Subtract the absorbencies for the solution at pH 4.0 from those of the alkaline solution.

The presence of morphine is indicated by the highly characteristic differences, having a maximum difference at 300 mμ, a minimum at 280 mμ, and another maximum at 255 mμ.

A quantitative estimation of the morphine in the sample can be made from the differences at 255 mμ.

(F) **Calculation.**—

Calculate the morphine in the sample from the following formula: Morphine in 50 c.c. of urine in mg. $= \dfrac{a \times b \times c \times d \times e \times f}{g \times h \times i \times j \times k}$.

(a) equals volume of solvent used to extract 50 c.c. of urine

(b) equals volume of solvent used to extract alkaline extract after adjusting the pH (125 c.c.)

(c) equals volume of alcohol used to dissolve residue (2 c.c.)

(d) equals volume of solvent used to extract the morphine liberated after destroying the iodoplatinate complex (25 c.c.)

(e) equals the amount of morphine standard in mg. (0.04 mg.)

(f) equals optical density difference at 255 mμ of the unknown

(g) equals aliquot of *a* (aliquot 1)

(h) equals aliquot of *b* (aliquot 2)

(i) equals aliquot of *c* applied to the filter paper strips

(j) equals aliquot of *d* (aliquot 3)

(k) equals optical density difference at 255 mμ of morphine standard (0.04 mg. = 0.68)

Morphine standard is 40 μg/c.c. (0.04 mg.) in 0.5N sodium hydroxide for the alkaline solution and in 0.5N sulphuric acid for the acid solution.

The recovery of known morphine concentrations added to urine is 75 ± 0.5%. Characteristic absorption spectra for morphine can be obtained with less than 0.1 mg. of morphine in 50 c.c. of urine.

DETECTION OF STRYCHNINE IN ANIMAL TISSUES

(A) Separation of Strychnine From Tissues, Food, or Contents of the Stomach

If a quantitative estimation is to be made, a weighed portion is selected. Divide into fine particles. Place in a flask, and cover with enough strongly acidulated distilled water to make a free-flowing mixture. Acetic or sulphuric acid is used.

Digest in the water-bath for about one hour.

Strain through gauze while hot.

Concentrate the liquid in the water-bath to about one-fourth its first volume.

Add to this concentrated liquid, while hot, five or six times as much strong alcohol. Stir the alcohol in slowly.

The alcohol precipitates a good deal of organic matter. Filter this organic matter out, washing the precipitate with portions of alcohol of the same strength as that of the filtrate.

Evaporate the whole filtrate on the water-bath to a syrupy liquid.

Cool.

Treat with enough water, slightly acidulated with acetic or sulphuric acid, to make it feasible to filter the whole into a separatory bulb or strong, large test tube.

Filter through filter paper into a separatory bulb or strong, large test tube. Render very slightly alkaline by adding potassium or ammonium hydroxide solution.

Shake out with chloroform. The shaking out is to be done thoroughly, but guard against making an emulsion.

Draw off the separated chloroform, and repeat a second and a third time.

Retain the chloroform extracts.

If the chloroform refuses to separate, add ether equal in volume to that of the chloroform. Centrifuging helps, but must be done cautiously. Chloroform alone is to be preferred.

Evaporate the combined chloroform extracts carefully on a water-bath. The residue contains the strychnine, if present in the original specimen.

To purify the residue further, take up with dilute acetic acid or sulphuric acid.

Filter.

Make alkaline, and repeat the chloroform extraction.

Add chloroform in a separatory tube.

Again evaporate the chloroform in a porcelain dish.

To purify further, add 2 or 3 drops of sulphuric acid.

Heat on a water-bath for one or two hours, or at 120° C. in a dry-heat oven for fifteen to twenty minutes. The residue becomes black.

Stir with water.

Filter.

Wash.

Make faintly alkaline.

Shake out with chloroform.

Concentrate the chloroform solution sufficiently, then keep in a well-stoppered tube for the testing.

(B) Qualitative Tests

The **biological test** is the most reliable. Frogs are selected.

Take up a portion of residue prepared by drying a portion of the chloroform extract on a water-bath.

Dilute in a small amount of sterile water or saline.

Inject into the abdominal sac of a frog. Note the time.

Keep the animal under a glass jar for observation.

Tetanic spasms are caused by strychnine, the spasms being preceded by a period of uneasiness with accelerated respiration. If tetanic spasms appear when the frog is stimulated, it points to strychnine or brucine. If convulsions set in, they may be due to a great number of poisons, or even to acidosis, brought about by having injected an acid solution of the organ or stomach extract. Acid or alkaline solutions themselves when injected may cause convulsions.[1] It is therefore imperative that the acid solution of the tissue extracts be neutralized with sodium bicarbonate before injection.

The frog test, if positive, must be followed by chemical tests for identification in medico-legal inquiries. If negative, then strychnine can be positively ruled out.

Wormley obtained, with frogs of from 15 to 50 grains (0.98 to 3.3 grams) in weight, from $\frac{1}{5000}$ grain (0.000013 gram), distinctive symptoms in from ten to thirty minutes; from $\frac{1}{500}$ grain (0.00013 gram), symptoms in three or four minutes, and death in from fifteen to thirty minutes. Kobert mentions $\frac{1}{30}$

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[1] Gettler, Alexander O.: Personal communication.

grain (0.002 gram) as a fatal dose for a frog. The paroxysms at first have remissions, and their return is hastened by agitation or vibration, as by striking on the table. Fatal dose is ½ grain or 0.032 gm. of strychnine or one of its salts.

A Rapid Bedside Test for Alkaloids

Method of Kaye

The procedure of Kaye has been found to be especially effective as a rapid means of detecting fairly large doses of various alkaloids.

Reagent.—

Dissolve 3.0 grams of red mercuric iodide
2.0 grams of potassium iodide
in 20.0 c.c. of glacial acetic acid.
Add enough distilled water to make a total volume of 60 c.c.
Keep the solution in a small brown dropping bottle.

Technic.—

Place 2 c.c. of urine in a small test tube.

Add 5 to 10 drops of the reagent.

An immediate turbidity which appears on slight agitation indicates the presence of either albumin or alkaloids.

Heat the tube. If the turbidity is due to albumin, the precipitate intensifies on heating, and persists.

If the turbidity is due to alkaloids, the precipitate completely dissolves upon heating and reappears upon cooling.

This method is very good when testing for alkaloids given in larger doses, such as, quinine, etc.

BAMFORD'S SYSTEMATIC SCHEME FOR THE IDENTIFICATION OF ALKALOIDS*

Group 1

Group Reagent.—Pure Concentrated Sulphuric Acid.

The following alkaloids yield definite colors:

1. Thebaine—immediately red, slowly turning orange.
2. Narceine—immediately greenish brown turning orange-yellow.
3. Narcotine—immediately lemon-yellow.
4. Hydrastine—green and brown patches, the whole slowly turning pink.
5. Solanine—yellow to yellowish brown.
6. Cytisine—orange, faint at first, becoming much deeper.
7. Piperine—reddish orange, turning brown.
8. Veratrine—yellow to orange-red, turning violet at the edges.
9. Delphinine—brown.
10. Colchicine—yellow.
11. Ergotoxine—yellow, becoming olive green at the edges and finally dark green.

Group 2

Group Reagent—Marquis Reagent (page 2307).—

This group contains alkaloids which give little or no colors with sulphuric acid alone, but give colors with the Marquis reagent.

Violet colors (various shades from bluish to reddish) given by morphine, apomorphine, heroin, Dilaudid, codeine, Dionin, eucodal, Dicodid.

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*Abstracted by permission from: Bamford, F.: *Poisons*, ed. 1, Philadelphia. The Blakiston Company. Pages 255-283.

Cherry red to reddish violet—lobeline.

These alkaloids are subdivided into groups 2a and 2b according to their behavior in Oliver's test. Group 2c consists of alkaloids which give colors with the Marquis reagent not closely resembling that given by morphine.

Violet with tinges of green—pseudomorphine.

Yellowish, becoming pale red-violet (not sensitive)—papaverine.

Light brown, becoming darker—ephedrine.

If a strong violet color is produced, Oliver's test should be applied. To a faintly acid solution of the alkaloid, add a drop of hydrogen peroxide and a drop of dilute copper sulphate solution. Then make the liquid alkaline with ammonia.

Group 2a—red to brown colors—morphine, apomorphine, heroin and Dilaudid.

Group 2b—no color—codeine, Dionin, Dicodid, eucodal or lobeline.

Group 2a

Colors with Oliver's Reagent

Morphine } red, quickly fading to brown
Apomorphine }
Dilaudid—yellow to brown
Heroin—bright red which persists for ½ hour.

Group 3

This group contains the alkaloids which respond to Vitali's test (below), or give colors with nitric acid and are not included in Groups 1 and 2.

Group 3a—atropine, hyoscyamine and scopolamine.

Group 3b—physostigmine, brucine, antipyrine and Pyramidon—these do not respond to Vitali's test.

Vitali Test.—Evaporate the alkaloid to dryness on a water bath with a drop of fuming nitric acid.

Treat the brownish residue, when cold, with a drop of alcoholic solution of potassium hydroxide.

The alkaloids of the atropine group, atropine, hyoscyamine, scopolamine (hyoscine) yield a strong bluish color.

Group 4

This group consists of the alkaloids which yield characteristic colors when dissolved in sulphuric acid and a minute crystal of potassium dichromate is drawn through the liquid.

Yohimbine—blue, changing blue violet and then to green
Strychnine—blue-violet, changing to red-violet, then to red and finally to orange.
Gelsemine—bright red, changing to brown
Curare—red-violet
Cotarnine—pink, changing to green
Hydrastine—salmon-pink, changing to brown

The group can be subdivided by use of Fröhde reagent.

Group 5

Group Reagent.—

Alcoholic solution of *p*-dimethylaminobenzaldehyde acidified with concentrated sulphuric acid.

The only natural alkaloids included are nicotine, mescaline, and pilocarpine, the other substances being artificial cocaine substitutes—Novocain, Anesthesin, Tutocaine, Pantocaine, and Stovaine.

All the substances in this group are local anesthetics—produce numbness when applied to tip of tongue.

The group is subdivided into the substances which yield colors in the cold and those of which the colors develop on evaporating just to dryness on the water bath.

Group 5a

Colors produced in the cold:

Strong yellow color—Novocain, Anesthesin, Tutocaine.
Pale yellow color—Pantocaine and Stovaine.

Effect of evaporating to dryness on the water bath—

Novocain, anesthesin and tutocaine become bright reddish orange.

Pantocaine—the color becomes strong lemon yellow.

Stovaine—the color becomes fainter and almost colorless.

Group 5b

Colors develop only on warming:

Blue violet at the edges, red-violet in the middle—nicotine.

Orange to red, rapidly fading on cooling—mescaline.

Strong yellow, pink at the edges—pilocarpine.

Group 6

This is a miscellaneous group of alkaloids all of which react with nascent chlorine to form products which are either colored themselves or become colored on treatment with ammonia.

Dissolve a few particles of the solid substance in a drop of concentrated hydrochloric acid, adding a minute quantity of potassium chlorate, and evaporating to dryness on the water bath. Note the color if any is produced and then expose the residue to ammonia fumes or touch with a glass rod moistened with ammonia—excess of ammonia should be avoided.

The following results are obtained:

Alkaloid	Color of dry residue	Color with ammonia
Caffeine	Red at edges	Purple
Quinine	Yellow at edges	Green
Quinidine	Yellow	Green or brown
Sparteine	Colorless	Reddish purple
Emetine	Pink (red before it is completely dry)	Brown
Percaine	Yellow	Olive green

Quinine and quinidine salts of oxyacids are strongly fluorescent in dilute solution.

Group 7

This group contains the alkaloids for which, with one exception, no satisfactory color reactions are known.

The group includes cocaine, tropacocaine, psicaine, alypin, cinnamyl-cocaine, α-eucaine, β-eucaine, aconitine, coniine, lupinine, and arecoline.

The first seven are local anesthetics and produce the characteristic feeling of numbness when applied to the tip of the tongue.

GROUP IV. HEAVY METALS

In testing for heavy metals, it is advisable to begin the analysis using fresh material. If history indicates general symptoms of metallic poisoning, that is, abdominal pain, vomiting, purging, metallic taste, bloody diarrhea, digest a definite weight of tissue or a definite volume of body fluid. When testing for a specific poison, it is far easier and more reliable to use the specific technic indicated for that poison and to select the organ best suited for this analysis. Consult Table 217 on page 2235.

Acid Digestion.—

For metals such as arsenic, lead, bismuth, antimony, thallium, etc.

Transfer 100 c.c. of urine to a 600 c.c. Kjeldahl flask.

Add 100 c.c. of nitric acid, c.p.

Then add 8 c.c. of sulphuric acid, c.p.

Heat the contents gently at first to prevent foaming. When the foam is broken, increase the heat and continue heating until the volume is approximately 20 c.c.

Allow to cool.

Add 50 c.c. nitric acid.

Then add (carefully) 5 c.c. of concentrated perchloric acid (72%).

Heat again until the solution clears and white SO_3 fumes are copiously evolved.

Continue heating for an additional several minutes. The solution is now completely digested and should be clear. Volume is approximately 6 c.c.

Allow to cool.

Dilute with distilled water to exactly 50 c.c. Final solution contains approximately 10% sulphuric acid. Tests for metals may be made on aliquots of this solution.

Spectrographic analysis of metals, especially for thallium and barium, is the best method of analysis. Transfer a small aliquot of acid digested material, above, to the crater of the carbon electrode and concentrate by complete drying. Attach to the spectrograph and analyze.

THE DETECTION OF ARSENIC

Spread the material in a thin layer in a large dish, and search carefully for white particles of arsenic. If these are found the test may be made on them. If not found, proceed with the extraction from the tissue.

(a) **Extraction from Tissues. Method of Taylor.—**

Cut the material into fine shreds.

Dry well in a water oven.

Pulverize the dried material in a mortar.

Stir the powder in strong, pure hydrochloric acid.

Set aside for twenty-four hours.

Distill through a Liebig condenser, heating the flask over a sand-bath. The distillate is collected in a flask containing distilled water. Material should be heated almost to dryness.

Treat a second time with hydrochloric acid.

Again distill.

(b) **Tests**

Marsh Test

In a flask suitable for generating hydrogen place several pieces of pure, arsenic-free zinc. Fit a two-hole rubber stopper in the flask. Through one of the holes place a thistle tube and through the other place a right-angle tube, which in turn is connected with a drying tube containing calcium chloride. Connect a piece of glass tubing, drawn out to make a very fine jet, with the drying tube. This tube should be bent upward at about a 60° angle. Through the thistle tube add dilute sulphuric acid (1 to 6 dilution) to the zinc. Hydrogen gas passes through the drying tube and is conducted through the jet. After a few minutes hold a test tube over the jet and collect some of the escaping gas. Carry the tube to a Bunsen flame and if it pops with the characteristic explosiveness of hydrogen, the apparatus may be considered safe to use. Ignite the gas escaping from the jet and hold a cold porcelain evaporating dish in the flame. If the chemicals are free from arsenic there will be no discoloration upon the dish and the unknown may be added to the flask. Add small amounts of the unknown solution through the thistle tube. If arsenic is present the flame becomes livid and more luminous. Hold a cold porcelain evaporating dish in the flame and if arsenic is present it will be coated, at the point of contact, with a black or deep brown spot, the typical ''arsenic mirror.''

Modified Gutzeit Method

Use of the modified Gutzeit apparatus, Fig. 690, page 2270, and Fig. 706.

Cut a disk of Whatman No. 40 filter paper. Impregnate with 5 per cent mercuric bromide in 95% alcohol for about 2 minutes. Shake off the droplets and fasten between the flanges of the apparatus using a rubber band. Prepare this just before beginning the test.

Into the small drying tube supported by the rubber stopper, plug some cotton moistened with lead acetate solution, above which the set of flanges is placed.

Place in the modified Gutzeit apparatus a few pieces of arsenic-free mossy zinc.

Add a few drops of dilute stannous chloride.

Add an exact aliquot of an acid digest solution of suspected arsenic (page 2324).

Quickly put the assembled flange in position in the flask.

The liberated hydrogen forms arsine on reacting with the deposited arsenic. Arsine travels upward and any impurity of hydrogen sulphide is removed by passing through the lead acetate moistened cotton. The arsine, upon contact with the mercuric bromide paper, turns the paper a yellow to brown color which is directly proportional to the amount of arsine produced.

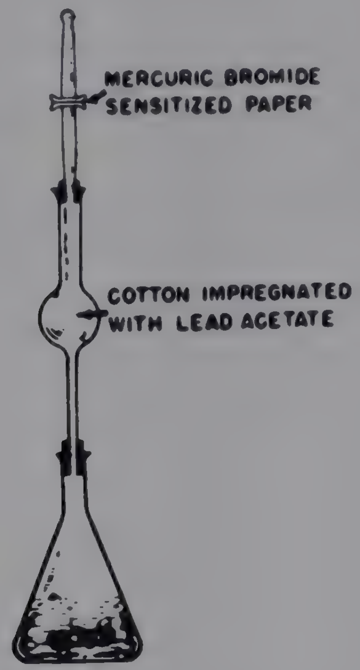
MERCURIC BROMIDE SENSITIZED PAPER
COTTON IMPREGNATED WITH LEAD ACETATE

Fig. 706.—The modified Gutzeit apparatus for detection of arsenic. The rubber stopper in the flask supports a small drying tube into which is plugged some cotton moistened with lead acetate solution. Above this tube is attached a set of flanges (Fig. 691) between which is placed a disk of mercuric bromide sensitized filter paper. The liberated hydrogen forms arsine on reacting with the deposited arsenic. Arsine travels upward and any impurity of hydrogen sulphide is removed by passing through the lead acetate moistened cotton. The arsine, upon contact with the mercuric bromide paper, turns it a yellow to brown color which is directly proportional to the arsine produced.

Approximately 30 to 40 minutes is sufficient time in which to liberate all of the arsine. To prevent possible interference of antimony, which may act similarly to arsenic under the conditions of the test, expose the canary yellow disk to hydrochloric acid fumes from an open bottle. The yellow color of antimony will completely fade, while the arsenic yellow color will persist.

This test is specific for arsenic and is sensitive to 0.005 mg.

For a quantitative analysis, prepare standards for comparison by making the test with various known quantities of arsenic, and saving the disks. The best range for comparison is made by using varying concentrations of arsenic from 0.005 to 0.040 mg. Compare the color of the disk from the specimen with the standard disks and record the result.

Reinsch-Gutzeit Test for Arsenic, Kaye Modification

This test is a further development of the Reinsch test for arsenic using the Gutzeit test as a confirmation for the arsenic deposit.

Apparatus.—

Copper Spiral.—

Make a copper spiral from 20 gauge copper wire, spiraled to about three-quarters of an inch in length. Clean with water, then alcohol, and finally with ether.

Gutzeit Apparatus.—

See Fig. 690, page 2270, and Fig. 706.

Reagents.—

Mossy Zinc, Arsenic-Free.—

5% Copper Sulphate Solution.—

Dissolve 5 gm. of copper sulphate, c.p., arsenic-free, in distilled water with a little heat. When cool, dilute to 100 c.c. with distilled water.

20% Stannous Chloride Solution.—

Dissolve 20 gm. of stannous chloride in 2 per cent hydrochloric acid and dilute to 100 c.c. with 2 per cent hydrochloric acid.

Mercuric Bromide Sensitized Paper.—

See pages 2271 and 2325.

Technic.—

Grind the material to be analyzed and place 10 grams of the ground material in a 50 c.c. Erlenmeyer flask.

Add 10 c.c. of distilled water.

Add 3 c.c. of concentrated hydrochloric acid.

Insert a copper spiral into the mixture, and heat on a steam bath for approximately 45 minutes, shaking occasionally.

Remove the copper spiral and wash it with distilled water. Darkening of the spiral may be due to arsenic, antimony, bismuth, silver, mercury, or even to prolonged heating. If only traces of any of these metals are present, the deposit might not be visible. In all cases, with or without a visible deposit, complete the determination.

Test for arsenic using the Gutzeit reaction, page 2325, but substituting the copper spiral for the acid digest solution.

Under these conditions only antimony and arsenic will produce the canary-yellow color which indicates a positive reaction. To remove any coloration which may be due to antimony, expose the strip or disk to hydrochloric acid fumes from an open bottle. The color due to arsenic will persist, while that due to antimony will disappear.

This reaction will detect as little as 0.010 mg. of arsenic in body fluids.

For quantitative tests, prepare standards as under the modified Gutzeit method, above.

When arsenic is deposited on the copper spiral, approximately one-half of the arsenic is deposited electrolytically, the other half forming a chemical reaction with the copper. With the Gutzeit test, therefore, only half of the arsenic is liberated. This has been proved experimentally. For a quantitative estimation of arsenic, multiply results by 2 after comparing with arsenic standards.

THE DETECTION OF MERCURY

Mercury is to be separated from the tissues and other organic matter by mincing them and heating on a water bath for some time with equal parts of water and hydrochloric acid, while potassium chlorate is added in small amounts until a clear solution is made. This is similar to the method described for lead. After filtration the solution is heated gently to expel the chlorine, and a stream of hydrogen sulphide is passed until the metal is all precipitated as sulphide. This may be tested by reduction and sublimation, or it may be dissolved by gentle heat in nitrohydrochloric acid, the solution evaporated to drying on a water bath, redissolved in warm water, and a test applied.

1. Sublimation Test for Compounds in the Solid State

The suspected solid is first thoroughly dried, mixed with dry sodium carbonate, and heated gently in a reduction tube. A shining ring forms on the inside of the tube in the cooler part. A lens resolves this sublimate into minute shining spheres of metallic mercury.

The corresponding sublimate of arsenic and antimony are not of this shape. Rubbed with a glass rod, these globules run together into larger rounded masses. A few scales of iodine left in the closed tube for a few hours will vaporize and convert the mercury into a film of yellow, and later of red, mercuric iodide. If this test is done in a small subliming cell, and collected on a slide for microscopic examination, it is of very great delicacy.

2. Hydrogen Sulphide Test

A solution of a mercurial salt acidulated with hydrochloric acid yields a black precipitate when treated with a stream of hydrogen sulphide. The formation of the mercuric sulphide through intermediate stages is shown if the tested solution is strong; the precipitate becomes successively yellowish white, dark yellow, orange, brown, and black. The precipitate is insoluble in caustic alkalies, alkaline sulphides, and nitric or hydrochloric acids. It can be identified by yielding the globular sublimate when dried and heated with sodium carbonate, as directed above. By drying the sulphide in an air-oven and weighing, the quantity of mercury may be calculated.

3. Reinsch's Test

A strip of bright pure copper-foil will receive a gray or silvery deposit in a few minutes from a boiling mercurial solution acidified with hydrochloric acid. After the coated copper is carefully washed in water and dried, the slip is heated in a small dry reduction tube, and the resulting sublimate is examined for globules and tested with free iodine. Certain of the organic mercurials may not readily respond to this test.

Fallacies.—This test yields a metallic deposit on copper from arsenic, antimony, bismuth, silver, and some rarer metals. Coatings of arsenic, antimony, and mercury are the only ones that give a sublimate when heated in a reduction tube.

Mercury is peculiar in its opaque globular form and the bright high lights under reflected light.

Delicacy.—Using capillary reduction tubes of peculiar construction, Wormley[1] has obtained characteristic globules from 1/500,000 grain of corrosive sublimate; under ordinary manipulation 1/100,000 grain is nearer to the limit of delicacy.

4. Potassium Iodide Test

On adding potassium iodide to a solution of corrosive sublimate or other mercuric salt, a precipitate falls, at first yellow, but rapidly changing to red mercuric iodide. This will dissolve in excess of the potassium iodide.

Quantitative Determination of Mercury

Since mercury is volatile, certain precautions are necessary when carrying out the digestion process. An aliquot of urine is digested with a reflux apparatus. Gettler and Lehman suggested the use of sulphuric acid and potassium permanganate. Ellmore suggested using fuming sulphuric acid, fuming nitric acid, and then potassium permanganate as digesting agents. With either method, after digestion is complete, the excess oxidizing agent liberated from the solution must be titrated with standard dithizone.

Reagents.—

Fuming Sulphuric Acid, 30% SO_3.—
Fuming Nitric Acid.—
Saturated Solution of Potassium Permanganate.—
Hydroxylamine.—
Ammonium Hydroxide.—
Dilute Sulphuric Acid.—

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[1] Wormley, Micro-Chemistry of Poisons, 1885, p. 204.

Standard Dithizone Solution.—

Dissolve 5 mg. of dithizone in 200 c.c. of carbon tetrachloride. Standardize as described on page 2346, substituting standard mercury solution for the lead solution.

Standard Mercury Solution.—

Dissolve 17 mg. of mercuric nitrate $Hg(NO_3)_2$ in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water. 1 c.c. contains 10 gammas of mercury.

Technic.—

Place 50 c.c. of urine in a flask.
Add 10 c.c. of fuming sulphuric acid.
Add fuming nitric acid, a few c.c. at a time, until the solution clears.
Connect the flask to a reflux condenser.
Heat for 20 minutes.
Cool, add water, and boil to remove all of the NO_2.
Add a slight excess of saturated solution of potassium permanganate.
Decolorize with hydroxylamine.
Make alkaline with ammonium hydroxide.
Very slightly acidify with dilute sulphuric acid.
Titrate with standard dithizone solution. The mercury-dithizone end point is orange. It can easily be detected after a little practice.

Calculations.—

Proceed with the calculations in the manner described for lead analysis on pages 2338 ff. and 2346.

A RAPID METHOD FOR THE ESTIMATION OF BISMUTH IN URINE[1]

Ever since bismuth was first introduced as an antisyphilitic agent, many methods have been developed and proposed for quantitative estimations in biological material. These methods include the well-known oxychloride test, the yellow color reactions with potassium iodide or thiourea, and the orange-red colored flecks produced when potassium iodide and cinchonine or quinine are added to a solution of bismuth. All of these methods, when applied to urine, require preliminary wet or dry digestion for the destruction of organic matter. The interference of other metallic ions such as lead, silver, copper, mercury, and iron have been pointed out by many workers.

The following method for the estimation of bismuth in urine may be used for the routine analysis of urine of patients under treatment with antisyphilitic therapy.

The essential features are: (1) depositing of the bismuth on a copper wire spiral (Reinsch test) under controlled conditions; (2) redissolving the deposited bismuth in dilute nitric acid in the presence of sodium sulphite. The interference of copper is eliminated by the addition of the sodium sulphite prior to redissolving the deposited bismuth in the dilute nitric acid. And (3) formation of bismuth-quinine-iodide and (4) the nephelometric comparison of this with known bismuth standards.

Reagents.—

Copper Wire Spirals.—

Make these from No. 20, B & S gauge wire. The spirals should be approximately one-fourth inch in diameter and three-eighths inch in length. They are easily made by winding the wire tightly and closely over a piece of glass rod and then cutting at intervals of 10 windings.

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[1] Kaye, S., and Castillo, J.: Am. J. Clin. Path. **14**: 7, 1944.

Hydrochloric Acid, c.p.—

Nitric Acid, 20 Per Cent by Volume.—

To 50 c.c. of distilled water in a 100 c.c. volumetric flask
add 20 c.c. concentrated nitric acid, c.p.
Dilute to 100 c.c. with distilled water.

Sodium Sulphite, 5 Per Cent Aqueous.—

Dissolve 5 gm. sodium sulph*ite*, c.p., in distilled water and dilute to 100 c.c. with distilled water.

Quinine-Potassium Iodide Reagent.—

Dissolve 1 gm. of quinine sulphate
in 100 c.c. of distilled water containing 0.5 c.c. of concentrated nitric acid.
When solution is complete, add 2 gm. of potassium iodide and dissolve.
Keep the solution in a brown bottle. It is stable.

Bismuth Standard Solution.—

Dissolve 0.1 gm. of metallic bismuth
in 10.0 c.c. of 35 per cent (1:1) nitric acid.
Dilute to 1000 c.c. with distilled water.
Store in a Pyrex bottle.
1 c.c. = 0.1 mg. of bismuth.

Standard Suspensions for Comparison.—

Use six small test tubes similar to those used in the test.
Place 1 c.c. of 5 per cent sodium sulphite solution in each tube.
Add 1 c.c. of 20 per cent nitric acid solution to each tube.

Add, respectively, 0, 0.1, 0.2, 0.4, 0.7, and 1.0 c.c. of the bismuth standard solution to the tubes. This corresponds to 0, 10, 20, 40, 70, and 100 gammas of bismuth, respectively.

Make the volume in each tube up to 3 c.c. with distilled water.
Add 1 c.c. of quinine-potassium iodide reagent to each tube and shake.

Technic.—

Thoroughly mix the twenty-four-hour specimen of urine to be tested.
Determine the total volume.
Place 10 c.c. of urine in a 50 c.c. Erlenmeyer flask.
Add 2 c.c. of concentrated hydrochloric acid.
Wash a copper spiral with alcohol, followed by ether, then dry.
Place the washed spiral in the flask and boil gently for 45 minutes.

Maintain the volume by adding 10 per cent (by volume) hydrochloric acid as required. If the mixture is boiled gently, approximately 5 c.c. of the dilute hydrochloric acid added every 15 minutes will suffice.

At the end of the boiling period, pour off the liquid, leaving the spiral behind.

Wash the spiral with distilled water by filling up the flask and pouring the water off as before. Repeat these washings several times.

Place the washed spiral on a piece of filter paper to remove any excess water.
Transfer to a small test tube (1 cm. diameter by 10 cm. in length).
Add 1 c.c. of 5 per cent sodium sulphite solution.
Add 1 c.c. of 20 per cent nitric acid solution.

Mix. Allow the mixture to act on the spiral, agitating frequently, until the bismuth which has been deposited on the spiral has dissolved. Approximately 5 minutes is sufficient time for deposit to be dissolved if the bismuth deposit is not too heavy.

Add 1 c.c. of distilled water
and 1 c.c. of quinine-potassium iodide reagent.
Shake and remove the copper spiral with the aid of a wooden applicator.

Compare the suspension of quinine-bismuth-iodide with the standard suspensions to determine the amount of bismuth present in 10 c.c. of urine. Calculate the amount in the twenty-four-hour specimen.

In using a copper spiral for the isolation of bismuth, interference of lead and iron is eliminated since these substances are not deposited on copper. Of the other metals which do form deposits on copper, namely, mercury, arsenic, antimony, and silver, none was found to give the double iodide with the reagent under the conditions of the test. Experiments were carried out to determine if the presence of these metals influenced depositing of bismuth on the copper. It was found that the presence of even as much as 50 gammas of any of these metals did not prevent depositing of bismuth on the spiral.

Although the degree of darkening of the copper spiral is a good indication of the amount of bismuth present in the urine, Kaye and Castillo found that the quinine-bismuth-iodide reaction detects deposits of bismuth not visible to the naked eye. Sensitivity for visible deposits is approximately 30 gammas. Sensitivity of the bismuth-quinine-iodide reaction is faint for 20 gammas and definite for 30 gammas of bismuth.

The number of standards is arbitrary. Where greater accuracy is desired than described here, a larger number of standards with a narrower range may be used. The upper limit of sensitivity, however, should be kept at 100 gammas because, with this test, differences in concentrations of bismuth above this level are difficult to estimate. Kaye and Castillo found that the best range for accurate estimations is between 20 and 70 gammas of bismuth in a 10 c.c. sample of urine. The total recovery of bismuth, as compared with the standards, is 80 to 90 per cent.

The copper spirals may be used for many determinations before being discarded if they are cleaned with dilute nitric acid after use.

If a more accurate quantitative determination is needed, first prepare an acid digestion of urine (page 2324). Then use an aliquot of this digest for determination with bismuth reagent as described above.

RAPID ESTIMATION OF MERCURY, BISMUTH, AND ARSENIC IN BODY FLUIDS (IN THE PRESENCE OF ONE ANOTHER)

Method of Kaye[1]

This is a rapid, simple, and reliable procedure for the microdetermination of mercury, bismuth, and arsenic in body fluids. Using the Reinsch principle, these metals can be isolated with rapidity and simplicity. Previous oxidation destruction of interfering organic materials is eliminated.

The Reinsch test previously served reliably only in a negative sense Various metals give similar appearances on the copper wire; prolonged heating of the organic material may also cause a similar tarnishing of the copper wire even in the absence of these metals. Several good confirmatory tests have been described to identify these metals when they are specifically indicated. A scheme of analysis for these metals in the presence of one another is, therefore, desirable.

Technic.—

Transfer a 20 c.c. or 20 gram aliquot sample of stomach contents, food, ground tissue, or concentrated urine to a 50 c.c. Erlenmeyer flask.

Add 4 c.c. of concentrated hydrochloric acid.

Introduce into the flask a 20 gauge copper wire, previously wound to a length of ½ inch and washed in alcohol and ether (page 2329).

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[1] Am. J. Clin. Path. **14**: 7, 1944.

Boil gently for 45 minutes.

Remove the spiral, wash it thoroughly with water, and dry it on absorbent filter paper.

A dark discoloration may indicate the presence of either bismuth or arsenic; discoloration is faintly visible in the presence of 0.030 mg. of bismuth and 0.020 mg. of arsenic.

Deposits due to mercury are usually grayish or silvery, faintly visible in amounts of 0.08 mg., definitely silvery in approximately 0.15 mg. amounts.

It is recommended that the procedures for identification be continued even in the absence of visible discoloration of the copper coil.

Identification and Estimation.—

1. Mercury

Cuprous Iodide Reagent.—

This is made according to the method of Ganassini.

Dissolve 5 gm. copper sulphate ($CuSO_4$)
and 3 gm. ferrous sulphate ($FeSO_4$) (anhydrous, precipitated with alcohol)
in 10 c.c. distilled water.

Add 7 gm. potassium iodide (KI) dissolved in 50 c.c. distilled water, stirring constantly.

Filter the precipitate and wash with distilled water.

Transfer the precipitated cuprous iodide to a brown bottle with the aid of a little water. Keep in the form of a suspension. It is quite stable.

Technic.—

Mercury can be quickly identified by the simple test described by Gettler.

Place a small filter paper on a watch glass.

Place 2 drops of a suspension of cuprous iodide on the filter paper.

Now place the copper spiral on the spot of cuprous iodide, and cover with another watch glass.

Allow to stand for 10 to 15 minutes.

A rose to salmon-pink color, due to the formation of mercury iodide, develops if mercury is present in amounts even as small as 0.025 mg.

By treating similarly known quantities of mercury, 0.025 mg., 0.050 mg., 0.10 mg., a set of standards may be prepared for matching colors produced by the unknown spiral: 0.025 mg. of mercury yields a very faint pink color, 0.050 mg., a more definite pink, and 0.10 mg. a distinct salmon-pink.

This test is specific for mercury; mercuric iodide is formed. All other metals which form deposits on copper when treated as outlined above give negative results with this test.

If a more accurate quantitative determination is necessary, once the presence of mercury is established, the dithizone procedure described on page 2329 is very satisfactory.

2. Bismuth

Wash the copper spiral free from any adhering cuprous iodide left from the previous test and allow it to dry on absorbent filter paper.

Transfer the spiral to a small test tube 1 cm. in diameter by 10 cm. in length.

Add 1 c.c. of 5 per cent sodium sulphite solution.

Add 1 c.c. of 20 per cent nitric acid solution.

Mix and allow to act on the spiral, agitating frequently until the deposited bismuth is dissolved. Approximately 5 minutes suffice if the deposit of bismuth is not too heavy. Arsenic will not dissolve under these conditions.

Add 1 c.c. of distilled water.

and 1 c.c. of quinine-potassium-iodide reagent (page 2330).

Shake and remove the copper spiral with the aid of a wooden applicator.

Save the spiral for the test for arsenic.

Compare the suspension of quinine-bismuth-iodide with standards described on page 2330.

This method is specific for bismuth under the conditions of the test. None of the metals which form deposits on copper give false positive reactions; the addition of the sodium sulphite prevents the copper iodide from reacting.

3. Arsenic

Wash the copper spiral again with water.

Prepare the modified Gutzeit apparatus as directed on page 2325. The flask contains some mossy zinc, arsenic-free, to which several drops of dilute stannous chloride solution and approximately 20 c.c. of 10 per cent sulphuric acid have been added. The sulphuric acid should be sufficient in quantity to cover the copper spiral completely.

Immediately connect the apparatus and allow 30 minutes for all the arsine to be liberated. An explanation of this method is given on page 2325. Differentiate from antimony as described on page 2326.

The test is specific for arsenic and is sensitive to 0.005 mg. Approximately only 50 per cent of the arsenic present can be recovered by this method because of the partial liberation of the deposited arsenic from the copper spiral. Kaye suggests that a factor of ×2 be used to compensate for this partial recovery.

For quantitative estimations, prepare standards covering the range of 0.005 to 0.040 mg. concentration of arsenic. Use these for comparison with the unknown.

Mercuric Bromide Paper.—

Cut Whatman No. 40 filter paper into disks.

Impregnate with 5 per cent mercuric bromide in 95% alcohol for about 2 minutes.

Shake off the droplets and fasten the disk between the flanges[1] of the modified Gutzeit apparatus, using a rubber band.

LEAD POISONING

The question of lead poisoning is a matter of great concern in connection with industry. Lead gains entrance to the body through inhalation, through ingestion by way of the gastrointestinal tract, and through the skin. Industrial exposure usually comes from inhalation of dust and fumes. Ingested lead may be excreted unchanged and even if absorbed may be carried into the liver and excreted into the bile.[2] Lanza[3] gives a complete story of the epidemiology of lead poisoning and states that the following summary refers to the main classification of industries and processes in which lead is a hazard:

1. Lead mining. In this case, lead is in the form of soluble carbonates or other soluble salts. If the ore is galena (lead sulphide), it is doubtful if lead poisoning occurs to any appreciable extent.
2. Lead smelting. Lead refining.
3. Fabrication and handling of lead:
    a) In the manufacturing of lead articles, poisoning is due to the formation of lead oxide on the surface of metallic lead.
    b) In lead burning, soldering, tempering lead, plumbing.

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[1] Flanges may be purchased from Eck and Krebb, New York City. Item No. 2080.
[2] Aub, J. C., Fairhall, L. T., and others: Medicine 4: 1, Feb.-May, 1925.
[3] J. A. M. A. **104**: 85, 1935.

c) Founding in which lead is used; for example, brass.

d) Buffing and polishing lead surfaces when lead is an ingredient of such substances so handled.

4. Manufacture of lead salts; for example, lead oxide by Dutch method, Carter method, etc.; lead pigments; organic lead compounds.

5. Manufacturing compounds which utilize lead: storage batteries, paints, rubber compounding, and chemical manufacturing.

6. In painting with lead paints, the application and removal thereof: painting, spray-painting, vitreous enameling, pottery, dipping, sandpapering, scraping and chipping painted surfaces. Flame cutting of painted metal. The spraying of trees with lead-containing insecticides.

7. In the typographic trades, such as, type founding, electrotyping, and stereotyping.

Lanza stated that when one comes across an outbreak of lead poisoning today, it is usually in an establishment where the lead process is but one step in the plan of manufacture. He also called attention to lead poisoning among industrial workers not engaged in handling lead but who, through negligence, are exposed to dust or fumes from lead processes not protected.

The amount of lead that is present in the atmosphere under varying conditions and the minimum dosage that will cause lead poisoning have been thoroughly studied. Bloomfield[1] reported that in a number of industrial establishments he found 0.10 mg. of lead to 10 cubic meters of air; in automobile repair shops, he found 0.13 mg., and in street air 0.09 mg. to 10 cubic meters. Greenburg[2] reported that exposure to 1.45 mg. of lead daily for two and one-half years will cause lead poisoning. Lanza states that in general lead in the form of vapor is more dangerous than as dust, and that lead in a molten condition gives off fumes; the higher the temperature, the more fumes. Oxide forms on the surface and gets into the air as dust. He calls attention to the fact that many lead cases are diagnosed as chronic appendicitis and even as gall bladder disease.

The biochemical behavior of lead in the blood is one of the most interesting aspects of lead poisoning. The work of Aub and others on this point has been already referred to. (See chapter on Hematology.) Aub[3] has epitomized the known facts on this subject. It was formerly thought that lead was carried in the blood stream as an albuminate, but present studies are rather convincing that lead is precipitated in the bones as the very insoluble tertiary lead phosphate and carried in the blood as the more soluble di-lead phosphate. Maxwell and Bischoff[4] have shown that lead, when injected intravenously, is carried as a diphosphoglycerate. Jowett[5] believes that it forms a complex inorganic phosphate with calcium and chlorine. No matter how it is transported in the blood stream, it causes deleterious effects on body cells.

Some absorbed lead is excreted without ever having been stored. There is a small daily excretion of lead in the urine in the vast majority of persons.

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[1] Bloomfield, J. J., and Isbell, H. S.: J. Indust. Hyg. 15: 144, 1933.
[2] Greenburg, L., Schaye, A. A., and Shlionsky, H.: Pub. Health Rep. 44: 1666, 1929.
[3] J. A. M. A. 104: 87, 1935.
[4] J. Pharmacol. & Exper. Therap. 37: 413, 1929.
[5] Biochem. J. 26: 2108, 1932.

the average excretion being from 0.05 to 0.1 mg. in the urine of normal persons daily. According to Barth,[6] a very small amount of lead may be gradually accumulated in normal bones during advancing years (from 0.02 mg. found in infancy to 0.1 mg. per 3 gm. of bone ash in the aged).

Absorbed lead is distributed throughout the viscera, but to the greatest extent in the liver, spleen and kidneys. A few days after absorption it collects in the bones. Aub and Minot* originally claimed that the direction of the lead stream is similar to that of the calcium stream or that, when calcium is being deposited in the bones, circulating lead is also deposited in the bones; and, when calcium is being pulled from the bones, some stored lead is also liberated. It is probably true that not all the calcium and lead thus liberated is excreted. X-ray evidence seems to indicate that it may circulate and be redeposited in bone, especially in childhood. Vogt shows by x-ray pictures that lead is repeatedly dissolved and redeposited along the epiphyseal line of growth. Aub states that because of this great avidity of growing bone for salts, as well as the difficulties precipitated by calcium deficiencies, deleading therapy seems an unwise and unsuccessful procedure in children.

The problem of treating lead poisoning is closely interwoven with these facts. If rapid deleading is to be avoided, one must simply give a large calcium intake in the diet, which lowers the demand on calcium stores, calcium is stored, and the only excretion of bone calcium and lead comes from the normal metabolism of the bones. This method should be used during any toxic lead episode.

Aub discussed in his work the problem of free deleading which should be undertaken after the episode has subsided and concluded as follows: (1) That lead is stored in relatively large quantities in the area of bone and is readily available for liberation; (2) that the liberation of this store is an obvious factor in the onset of toxic lead episodes during metabolic upsets; and (3) that this liberated lead may not be excreted but may circulate and be redeposited. (No one knows the extent of this possibility.)

In favor of deleading we have the possibility, under controlled conditions, of reducing the lead contamination in the bone trabeculae, then replenishing the trabeculae with uncontaminated calcium. Against deleading is the consideration that it may not be desirable to liberate the lead, but to keep it stored, largely in the bones, during good health. Theoretically, deleading appears advantageous in that it would seem to avoid sudden liberation of this lead in time of metabolic stress. Practically, that procedure should be followed which will most promptly return the patient to health. Aub has found that following the ordinary toxic lead episode a vigorous course of deleading usually is followed by prompt return to normality, and that this recovery is more rapid and much more complete than when a continued deposit of lead stores is maintained. He believes that in lead palsies thorough deleading approximately halves the period of disability. Although this opinion may possibly be altered, Aub stated that the biochemical knowledge on which this treatment is based seems to be thoroughly established, not only for lead but also for several other related heavy metals.

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*Aub, J. C., Fairhall, L. T., Minot, Anne S., and Reznikoff, Paul: Lead Poisoning, Medicine Monographs, Baltimore, Williams & Wilkins Company, 7, 1926.

[6] Virchows Arch. f. path. Anat. 281: 146, 1931.

The question of the normal absorption and excretion of lead has been thoroughly studied by Kehoe, Thamann, and Cholak[*] They studied the incidence of lead ingested normally and lead excreted. They found that normal lead found in the excretions came largely from food. They found that the mean daily lead content of twenty-four samples of food was approximately 0.25 mg. and the mean daily fecal output amounted to about 0.26 mg. They found that in twenty-four-hour samples of normal feces, there might be 1 mg. of lead, while in a normal sample of urine, we might find as much as 0.20 mg. per liter rarely and 0.1 mg. per liter not uncommonly. They warn against the danger of interpretation from any single analytical result, and state that in normal individuals not even exposed to lead, there should be repeated examinations of urine and feces before diagnosis is established.

DETERMINATION OF LEAD IN BIOLOGIC TISSUES AND FLUIDS

Determination of Lead by Dithizone

Bambach (Modification of the Hubbard-Clifford-Wichmann Method)

The method by Bambach[1] is an improvement of the method reported by Hubbard,[2] who based his photometric dithizone procedure upon work done by Clifford and Wichmann,[3] and by Wilkins, Willoughby, Winter, and their co-workers.[4-6] These improvements have made possible increased accuracy and the saving of time.

Apparatus.—The apparatus used by Bambach is essentially the same as that described by Hubbard. This is a neutral wedge photometer, as described by Clifford and Wichmann, with slight modifications.

TABLE 231.—ANALYSIS OF REAGENTS COMMONLY USED IN DITHIZONE METHOD

REAGENT	LEAD CONTENT GAMMA/LITER
Water (regular double-distilled, Barnstead still)	2
Water (triple-distilled, third distillation in Pyrex)	Trace[a]
HNO_3 concentrated	10-30
HNO_3 concentrated (triple-distilled, in quartz)	Trace[a]
HCl concentrated	10-30
HCl constant-boiling (triple-distilled, in quartz)	Trace[a]
H_2SO_4 concentrated	Ca. 800
KCN solution, 10 per cent W/V	80
Ammonium citrate solution, 40 per cent W/V	Trace[a]

[a] Less than 1 gamma.

Light Box.—The light source consists of nine 32-candlepower, 12- to 16-volt, automobile headlight bulbs (Mazda ''Tung Sol'' T-1144) wired in series from a 122-volt alternating current circuit and arranged in a circle around two 2.5 cm. (1-inch) circular apertures in the interior of the box. The light is reflected from a piece of solid opal glass 15 × 15 cm. (6 × 6 inches) mounted about 2.5 cm. (1 inch) from the light source, so as to give diffuse reflected light.

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[*] J. A. M. A. **104:** 90, 1935.

[1] Ind. & Eng. Chem. **11:** 400, 1939.

[2] Ind. & Eng. Chem. **9:** 493, 1937.

[3] J. Assn. Official Agr. Chem. **19:** 130, 1936.

[4] Wilkins, E. S., Jr., Willoughby, C. E., Kraemer, E. O., and Smith, F. L.: 2nd Ind. Eng. Chem., Anal Ed. **7:** 33, 1935.

[5] Willoughby, C. E., Wilkins, E. S., Jr., and Kraemer, E. O.: Ibid. **7:** 285, 1935.

[6] Winter, O. B., Robinson, H. M., Lamb, F. W., and Miller, E. J.: Ibid. **7:** 265, 1935.

Eyepiece.—Prism system and housing complete, from the Bausch & Lomb No. 3600 hemoglobinometer.

Wedge.—A neutral density wedge constructed of Bausch & Lomb smoked "C" neutral glass, length 150 mm., height 20 mm., thickness at thick end 8.0 mm. and at thin end not more than 0.5 mm. The wedge is compensated and reinforced by a wedge of clear glass of the same dimensions cemented to the wedge proper.

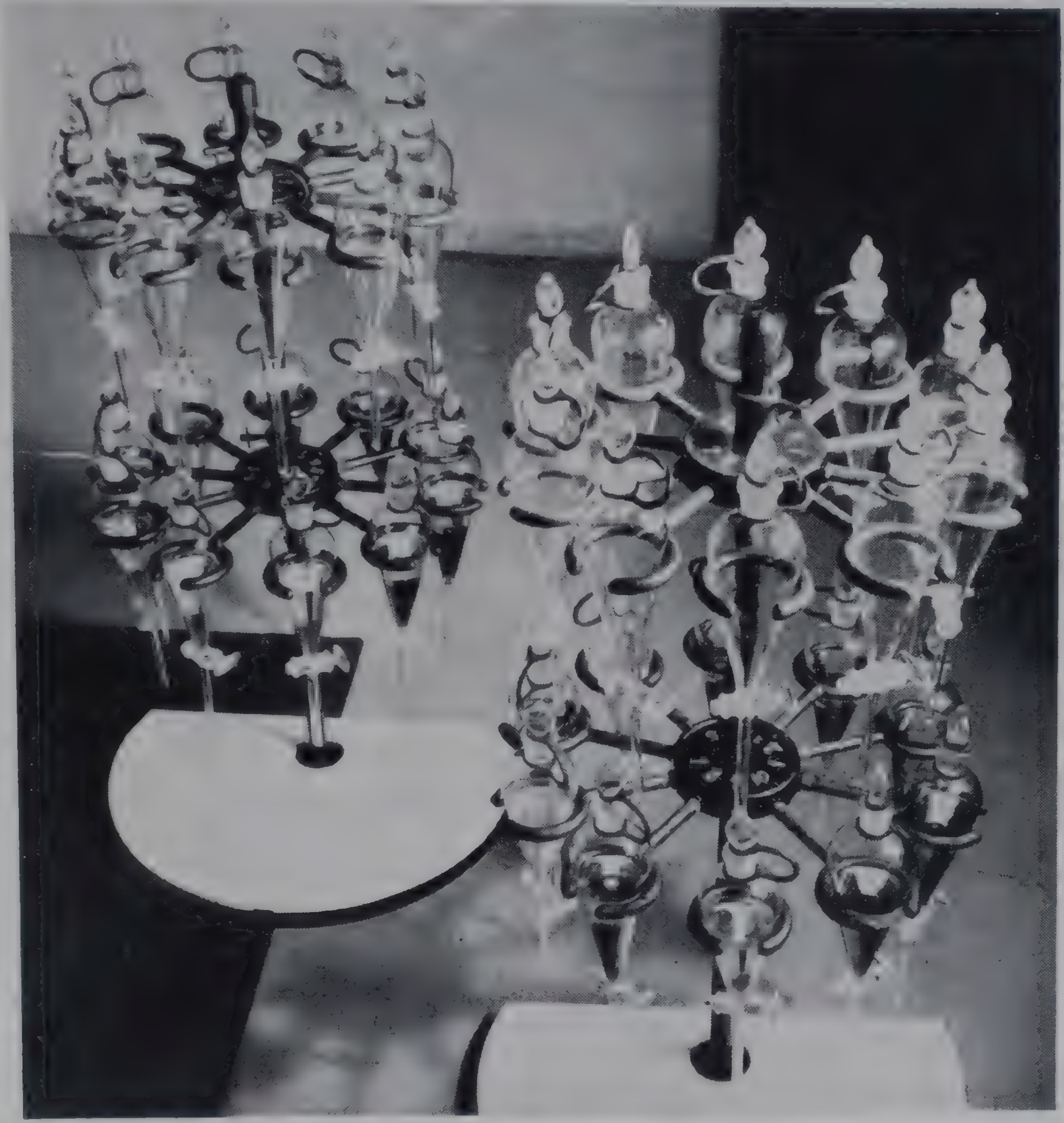

Fig. 707.—Rotary funnel racks, dithizone determination of lead. (Bambach, Karl: J. Ind. & Eng. Chem. July, 1939.)

Cells.—All cells are constructed of Pyrex glass with ground and polished fused-on windows of Corex glass. The windows are parallel and optically plane. No flux or cement is used in the construction. Vents are placed in the tops of the cells to permit of ready emptying and filling. Three cells are used having internal lengths of 12, 25, and 50 mm. (tolerance of ±0.1 mm.) for ranges of 0 to 10, 0 to 50, and 0 to 100 gamma of lead, respectively. The internal diameter for these cells is 14.5 mm. with a tolerance of ±0.5 mm.

Filter.—The spectral region employed (510 mμ) is obtained by means of a Wratten compound filter (Eastman Kodak No. 45 and No. 58). The maximum transmission of this filter (10 per cent) occurs at 510 mμ and it decreases sharply for regions on either side of the maximum, reaching a value of only 0.1 per cent at 478 and 550 mμ.

Bambach introduced a neutral wedge photometer improved with a few changes. A small compensating wedge of Bausch & Lomb neutral "C" glass was placed in the light path, as suggested by Clifford, in order to make the comparison fields of the instrument ''shade off'' similarly. In addition, the compound gelatin filter has been replaced by a permanent glass filter with maximum transmission at 510 millimicrons. These changes necessitated a stronger light source and accordingly 50-candlepower bulbs have been substituted for the 32-candlepower bulbs originally used.

A special device, suggested by R. R. McNary, of the Kettering Laboratory of Applied Physiology, University of Cincinnati, is advised. This consists of special rotary funnel racks fitted with opaque glass bases. The metal rings which hold the funnels have been rubber-plated by the anode process. This rubber covering resists the reagents used in the analysis much better than any type of paint available (Fig. 707).

Complete Procedure for Lead Determination*

Preparation of Samples

Urine.—

Add concentrated nitric acid (10 per cent of the sample volume) to the measured sample. Evaporate to dryness on a hot plate or steam bath, and ignite at 500° C. in a silica or Pyrex evaporating dish. Moisten the ash with nitric acid, dry, and ignite for a few minutes until it is white; dissolve in dilute nitric acid and the sample is ready for analysis. Filtration is not necessary.

Feces.—

Dry the sample on a hot plate or steam bath to constant weight in a tared silica or Pyrex dish. Place the dish on an electric heater at a low red heat to drive off volatile matter which would otherwise flame in the muffle furnace. After constant weight is attained ignite at 500° C. in a muffle furnace. Weigh the ash and dissolve it in a mixture of hydrochloric and nitric acids. The sample is now ready for analysis. Filtration is not necessary.

Foods.—

Digest the weighed sample of mixed food with concentrated nitric acid on a hot plate or steam bath until all lumps have disappeared. This may require over a week, with repeated additions of acid. If a food homogenizer, such as a Waring Blendor, is used, the time may be materially shortened, especially since aliquots may be employed. Evaporate the sample to a syrupy consistency, transfer to a silica or Pyrex evaporating dish, and add concentrated sulphuric acid (15 c.c. for a normal day's mixed food). Place the dish on an electric heater at a low red heat and treat as described under ''Feces.''

Tissues.—

Weigh the sample and treat in the same general way as a sample of mixed food.

Blood.—

Transfer the sample to a tared silica or Pyrex evaporating dish, weigh, and add concentrated nitric acid (5-10 c.c. per 10 gm. of blood). Evaporate the mixture to dryness on a hot plate or steam bath, place on an electric heater at a low red heat, and ignite in a muffle furnace at 500° C. Add a few c.c. of hydrochloric acid to the ash, then nitric acid, and heat the dish on the hot plate until the sample is dissolved. It is then ready for analysis.

Alternate Procedure

The perchloric procedure is an alternate method for digesting any of the biological materials discussed above.

Place in a 300 c.c. Pyrex Kjeldahl flask 10 c.c. of concentrated sulphuric acid.

Add 100 c.c. of concentrated nitric acid. The quantity of these acids will vary with the quantity and nature of the biological material digested.

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*Personal communication: William E. Crutchfield, Jr., Kettering Laboratory of Applied Physiology, College of Medicine, Cincinnati, Ohio.

Weigh the specimen.

Introduce a small quantity into the Kjeldahl flask.

Heat gently at first to retard foaming. Increase the heat and continue heating until the volume is approximately 20 c.c.

Cool the solution and add an additional 100 c.c. of concentrated nitric acid and 5 c.c. of concentrated perchloric acid (72%).

Heat again, continuing until the solution is completely digested and copious white fumes of sulphur trioxide begin to evolve. Solution is now approximately 5 c.c.

Allow to cool.

Add 3 c.c. of 5 per cent hydrochloric acid saturated with sodium chloride.

Heat again to dissolve the lead sulphate precipitate.

Filter while hot.

Wash the filter paper with several 5 c.c. portions of *hot* distilled water.

The solution is now ready for analysis.

Technic.—

Note: All glassware should be cleaned with sulphuric acid-chromic acid mixture and with dilute nitric acid (1 + 1). Careful attention is necessary to avoid dust contamination, especially during the evaporations. If reagent acids are used in the sample preparations, lead determinations should be made on them for deduction as reagent blanks. This can be eliminated by using distilled acids.

Reagents and Apparatus.—

Ammonium Citrate Solution.—Dissolve 400 grams of citric acid in water with sufficient reagent ammonium hydroxide added to make the solution alkaline to phenol red. Dilute the solution to one liter with water and purify by shaking it with repeated portions of a solution of dithizone in chloroform until the dithizone retains its original green color.

Distilled Ammonium Hydroxide.—Distill reagent ammonium hydroxide into double-distilled water chilled in an ice bath.

Hydroxylamine Hydrochloride Solution.—Dissolve 20 grams of hydroxylamine hydrochloride in sufficient water to make about 65 c.c. and add a few drops of m-cresol purple indicator solution. Add concentrated ammonium hydroxide until a yellow color results. Add sodium diethyldithiocarbamate in water (an approximately 4 per cent solution) in sufficient quantity to combine with all the lead (and most other metals) present and to leave a considerable excess. After a few minutes completely extract the organo-metallic complexes and the excess reagent with chloroform. (The absence of a yellow color in the chloroform when a portion of the chloroform extract is shaken with a dilute solution of a copper salt indicates when this point is reached.) Add distilled hydrochloric acid to the hydroxylamine hydrochloride solution until the indicator turns pink, and add double distilled water to make the final volume 100 c.c. It is not necessary to filter the solution.

Potassium Cyanide Solution.—Shake a substantially saturated solution containing 50 grams of potassium cyanide in sufficient water to make 100 c.c. with portions of dithizone in chloroform (30 mg. per liter) until the lead is removed. Part of the dithizone dissolves in the aqueous phase but sufficient remains in the chloroform to color it and to indicate when the lead has been completely extracted. Most of the dithizone in the aqueous phase can be removed, if desired, by repeated extractions with pure chloroform. Dilute the strong potassium cyanide solution with double distilled water to the proper strength (10 grams per 100 c.c.). It is not necessary to filter the solution. (If, instead of the concentrated solution, the final one is shaken with dithizone in chloroform in an attempt to delead it, the increased alkalinity of this dilute solution causes the removal of all the excess dithizone from the chloroform and renders the complete extraction of the lead more difficult.)

Dithizone Extraction Solution.—Shake one liter of chloroform with 100 c.c. of water containing about 0.5 gm. of hydroxylamine hydrochloride which has been made alkaline to phenol red with ammonium hydroxide. Drain the chloroform off, and dissolve 30 mg. of dithizone in the chloroform. Add approximately 5 c.c. of alcohol to the solution if part of it is to be kept for several days. Filtration is not necessary. Shake the quantity of dithizone solution to be used for one day with about 100 c.c. of dilute nitric acid (1 c.c. per 100 c.c.) just before use.

Standard Dithizone Solutions.—Make these solutions from distilled chloroform which has been treated with hydroxylamine as in the instructions for the dithizone extraction solution. In this case, however, filter the chloroform through a dry paper into a glass-stoppered Pyrex bottle, which is protected from light by being wrapped with heavy paper or by a wooden box. Dissolve the proper amount of dithizone (5 mg. per liter for the 0-10 microgram range, 10 mg. per liter for the 0-50 microgram range, and 20 mg. per liter for the 0-100 microgram range) in the chloroform, add absolute alcohol (5 c.c. per liter) and the solution is ready for standardization. It should be kept in a refrigerator.

Ammonia-Cyanide Mixture.—Each liter of the mixture contains 20 gm. of potassium cyanide and 150 c.c. of distilled ammonium hydroxide, specific gravity 0.9, or its equivalent; dilute to volume with double distilled water. This solution should be kept in a cool place.

Buffer Solution, pH 3.4.—Dilute 9.1 c.c. of reagent nitric acid to approximately 500 c.c. with double distilled water in a one-liter volumetric flask, add bromthymol blue indicator, and bring the pH to 3.4 with distilled ammonium hydroxide. Add 50 c.c. of Clark and Lubs potassium acid phthalate-hydrochloric acid buffer, pH 3.4, double strength (50 c.c. M/5 potassium acid phthalate, 9.95 c.c. M/5 hydrochloric acid, per 100 c.c.) and dilute the solution to one liter with double distilled water.

Chloroform Recovery.—Collect used dithizone solution in glass-stoppered amber bottles containing alcohol (about 0.5% of capacity of bottle). Wash each liter of solution with about 200 c.c. of water, then with two 100-c.c. portions of concentrated sulphuric acid. Add 5 c.c. of alcohol to the chloroform and allow it to stand overnight in the dark over lump calcium oxide. Distill through a still, using all-glass connections into a glass-stoppered amber bottle containing absolute alcohol (about 1% of capacity of bottle).

Apparatus.—Pyrex glassware should be used throughout; containers should all be glass-stoppered. The extractions are most conveniently carried out in Squibb-type separatory funnels, 150 c.c. capacity, graduated at 5 c.c., 10 c.c., and 10 c.c. intervals to 100 c.c., using the rotary funnel racks previously described. Use white petrolatum to grease the stopcocks, since it is practically lead-free.

Any photometer which can be used with the cells described is usually satisfactory for this procedure. A glass filter with maximum transmission at 510 mμ is suitable. The neutral wedge photometer described by Clifford and Wichmann was originally used in this work.

Wash all glassware (Pyrex) thoroughly before use with hot 50 per cent nitric acid, and rinse with distilled water to ensure the removal of lead which may be present as surface contamination. Use the same procedure for silica or porcelain ware.

Isolation of Lead.—Pour approximately 15 c.c. of the ammonium citrate solution into each of the lower funnels, using the graduation marks on the funnels as guides. Add the proper aliquots of the prepared samples (equivalent to 50-200 c.c. of urine, 0.1-0.5 gm. of fecal ash, 5-20 gm. of blood, or 1/10 of a normal day's food), with 5 c.c. of potassium cyanide solution and 1 c.c. of hydroxylamine hydrochloride solution, and make the mixtures alkaline to phenol red with distilled ammonium hydroxide. In rare instances the solutions will become very cloudy upon being made alkaline, due to the presence of an unusually large quantity of calcium phosphate; in that case add sufficient ammonium citrate solution to dissolve the precipitate.

Begin the extraction of lead with 5 c.c. of dithizone extraction solution and note the color in order that the proper standard dithizone solution may be chosen in the final lead estimation. (The lead usually is not extracted quantitatively with each portion of dithizone solution because of the various salts present. Instead of 50 micrograms of lead, which is the theoretical equivalent of the dithizone, usually only 40 micrograms will be extracted by each 5 c.c. When the quantity of lead present is less than 10 micrograms this is recognized by the distinctive greenish-blue color of the dithizone.)

Add another 5 c.c. portion of dithizone solution using the graduation marks on the funnels as guides, and note the color of this combined 10 c.c. solution before it is drained into the other funnel. This color should indicate whether the quantity of lead present is greater than 50 micrograms, or greater than 100 micrograms. Continue the extraction with successive 5-c.c. portions of dithizone solution, noting the color of each portion. Drain each portion before adding the next, until the lead is completely extracted.

Wash the combined dithizone extracts with approximately 50 c.c. of double distilled water and wash the water with 5 c.c. of distilled chloroform. This chloroform washing should be green in color; if it is not, the presence of lead or zinc is indicated. If such is the case a drop of potassium cyanide solution should then be added to the water and the funnel shaken again; if the chloroform phase does not become green the water should be washed at least once with dithizone extraction solution. Add all chloroform washings to the dithizone extract and discard the water. Strip the lead from the dithizone extract by shaking with 50 c.c. of buffer solution (pH 3.4); if the dithizone solution does not return to its original green color, bismuth is present. Drain dithizone solution from the funnel; and if the sample contains bismuth, shake the buffer solution with one 5 c.c. portion of dithizone solution. After this is drained out, add 5 c.c. of distilled chloroform and shake the mixture.

In the event that the sample taken contains over 100 micrograms of lead, as indicated by the color of the separate portions of dithizone extraction solution early in the lead isolation step, discard a part of the buffer solution containing the lead before adding the 5 c.c. of distilled chloroform. The quantity discarded should be sufficient to reduce the lead below 100 micrograms; this is conveniently done by using the graduation marks on the funnel. Return the volume to 50 c.c. by adding more buffer solution and add the chloroform as indicated previously. Allow the funnel to stand unstoppered until the drop of chloroform floating on the surface of the aqueous phase has evaporated and draw off the 5 c.c. of chloroform as completely as possible without allowing water to enter the bore of the stopcock.

Final Estimation of Lead.—In the following part of the procedure direct sunlight should not be allowed to strike the solutions.

Add the proper standard dithizone solution (10 c.c. of the 0-10 microgram solution, 25 c.c. of the 0-50 or 0-100 microgram solution) to the funnel containing the lead in 50 c.c. of buffer solution. Add 7 c.c. of ammonia-cyanide mixture, and shake the funnel immediately for one minute. The pressure which develops should not be released through the stopcock; instead the stopper should be lifted in order to avoid blowing water into the funnel stem. If a number of analyses are being run, the mixed color can be developed in the whole series and photometric readings taken one after the other.

Use part of the dithizone solution (2 c.c. of the 0-10 microgram solution, 10 c.c. of the 0-50 or 0-100 solution) to flush the stem of the funnel, dry the end of the funnel stem, and allow the solution to run directly into the proper cell for the photometric reading. Use the 0-10 microgram solution with a 2-inch cell, the 0-50 microgram solution with a 1-inch cell and the 0-100 microgram solution with a ½-inch cell. The cells are cylindrical, with optically plane ends, and have an internal diameter of about 14 mm. Since the 0-10 microgram cell holds the entire 8 c.c. remaining in the funnel, it cannot be rinsed with part of the dithizone solution, but must be cleaned and dried with pure acetone after each sample. The other two cells, however, can be rinsed with dithizone solution at least twice and it is rarely necessary to wash or dry them between samples.

The photometric readings are referred to calibration curves made for each standard solution in the following way:

Make a standard lead nitrate solution by dissolving recrystallized lead nitrate in 1% nitric acid, so that each c.c. contains 1 mg. of lead. From this solution make two dilutions in 1% nitric acid, one containing 10 micrograms of lead per c.c., and the other 1 microgram of lead per c.c. All these solutions will keep indefinitely in glass-stoppered Pyrex containers.

Make a standard lead solution in the proper buffer by taking a measured quantity of the desired standard lead solution, bring it to pH 3.4 by the addition of dilute distilled ammonium hydroxide. Add the proper amount of the Clark and Lubs buffer mentioned above, and dilute the mixture to a known volume. This solution should be made fresh each time it is used.

Add measured quantities of this standard lead solution to clean separatory funnels, bring the volume in each funnel to 50 c.c. with the pH 3.4 buffer solution and add the dithizone solution which is to be standardized. Carry out color development and photometric readings as described under the heading "Final Estimation of Lead." From these readings make a standard calibration curve.

Rapid Microanalytical Test for Lead in Urine

Method of Kaye[1]

Aub and his associates showed that ammonium hydroxide quantitatively precipitates lead from urine in the form of lead phosphate. It has been shown experimentally, however, that 100 per cent recovery of lead does not occur when ammonia is added to the urine in excess. Fairhall stated that 100 per cent precipitation of lead from urine occurs with ammonia provided the reaction of the urine is brought to pH 10.0 with ammonia. This fact has been proved experimentally.

Several excellent procedures have been described for the destruction of organic matter in urine preparatory to the determination of lead. These use either dry muffle heat below 500° C. or various acid oxidation modifications. Although these methods are fairly rapid, they require more time than the phosphate precipitation method. Moreover, when the wet oxidation methods are used, the quantities of urine analyzed are so small that slight deviations become marked and significant when calculations are made on the basis of a liter of urine per day. Larger samples of urine may, of course, be digested in carrying out these tests, but this increases the time required for analysis.

Principle.—

The method is based upon the total precipitation of lead by the addition of ammonium hydroxide to urine. Lead phosphate is formed. It is very insoluble and easily separated by filtration along with the alkaline earth phosphates of calcium and magnesium. This precipitate is subsequently dissolved with hot dilute nitric acid and is heated for a short time to oxidize any organic material which may have adhered to it. Citric acid is added to keep the phosphates in solution during subsequent neutralization. Potassium cyanide is added to form complex ions with interfering metals.[2] The reaction is adjusted to pH 9.0 to 10.0 and the lead is extracted with dithizone to separate it from extraneous salts and interfering substances. Once separated, the lead is split from the dithizone and then titrated with standardized dithizone for an accurate determination. With this test, only bismuth, tin, thallium, and lead react with dithizone. Interference of bismuth and tin is eliminated by previous extraction with dithizone while the solution is still acid. Lead is not extracted in this acid solution. Thallium, as a source of interference, has not been removed, but its occurrence is rare, and it is not likely to be found.

Reagents.—

All reagents must be kept in Pyrex glass bottles. Use distilled water from an all-Pyrex still. Use ammonia water and nitric acid with low lead content.

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[1] J. Lab. & Clin. Med. 28: 9, 1943.

[2] Cd, Zn, Cu, Co, Ni.

Phenolphthalein Indicator.—

See page 1199.

Ammonium Hydroxide (Low Lead Content).—

8% Nitric Acid.—

Add 80 c.c. of nitric acid, c.p. (low lead content) to distilled water, and dilute to 1000 c.c. with distilled water.

10% Potassium Cyanide Solution.—

Dissolve 10 gm. potassium cyanide (low in lead) in distilled water and dilute to 100 c.c. with distilled water. POISON.

40% Citric Acid Solution.—

Dissolve 200 gm. citric acid (low in lead) in distilled water and dilute to 500 c.c. with distilled water.

Standard Lead Solution.—

Dissolve 160 mg. of lead nitrate in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

1 c.c. is equivalent to 100 gammas of lead.

Dithizone 1 for Preliminary Extraction.—

Dissolve 25 mg. of dithizone in 400 c.c. of chloroform.

Dithizone 2 for Titration.—

Dilute 100 c.c. of dithizone 1
with 100 c.c. of chloroform.

Standardize against a known lead solution (see below).

The approximate value is 1 c.c. = 5 gammas of lead.

If kept in a brown bottle and in a cool place, the titer will not change for several months. This titer is sastisfactory for amounts of lead near normal values. If larger amounts of lead are apparently present, as determined by the amount of dithizone required for preliminary extraction, a solution with stronger titer may be prepared. When dithizone 1 is standardized, the titer is approximately 1 c.c. = 10 gammas lead. The titer should be calculated to at least one decimal unit.

Technic.—

Collect and measure the total 24-hour specimen of urine.

Remove an exactly measured aliquot for analysis, usually 300 c.c.

Add a few drops of phenolphthalein indicator.

Add ammonium hydroxide, a drop at a time, until the urine turns pink. The reaction is now pH 9.0 to 10.0.

Stopper, shake well, and allow the urine to stand for about 30 minutes until the precipitate which forms settles out.

Separate the precipitate by filtering through a Buchner funnel using suction. Refilter if necessary.

Wash the precipitate with several small portions of alcohol.

Invert the filter paper so that the precipitate faces the perforations of the funnel, and insert the funnel into a clean flask.

Pour approximately 30 to 50 c.c. of hot 8 per cent nitric acid over the precipitate, repeatedly, until the precipitate either dissolves or is completely transferred to the flask. This is more easily accomplished without suction.

Boil the total redissolved precipitate gently for approximately 30 minutes until the foaming stops. This indicates that the proteins are destroyed. If the volume has decreased greatly, restore it with lead-free distilled water.

Adjust the reaction to pH 3.0 to 3.5, the lower range of methyl orange, by adding dilute ammonium hydroxide a drop at a time until the proper color develops.

Table 232.—Distribution of Lead in Human Tissues at Various Intervals Following Cessation of Lead Exposure

IDENTIFICATION	A. J.		J. G.		A. S.		E. Mc.		E. J.		B. X.	
AGE AND WEIGHT	3 yr.—10 kg.		54 yr.—55 kg.		29 yr.—60 kg.		58 yr.—70 kg.		25 yr.—80 kg.		4 yr.—12 kg.	
NATURE OF LEAD EXPOSURE	Short period of ingestion		Severe dust inhalation—several yr.		Severe dust inhalation—6 yr.		Moderate dust inhalation—20 yr.		None		None	
INTERVAL BETWEEN EXPOSURE AND DEATH	Days?		1 month?		8 months		2 years		-		-	
TISSUE	Mg. found	Mg./100 grams	Mg. found	Mg./100 grams	Mg. found	Mg./100 grams	Mg. found	Mg./100 grams	Mg. found	Mg./100 grams	Mg. found	Mg./100 grams
Kidneys	0.65	0.61	0.65	0.22	0.00	-	0.16	0.06	0.21	0.07	0.07	0.06
Liver	16.00	3.27	9.20	0.71	0.88	0.31	1.46	0.12	1.75	0.08	0.22	0.05
Spleen	0.54	1.25	1.29	0.86	0.05	0.10	0.10	0.10	0.00	-	Trace	-
Fat	-	-	0.00	-	-	-	-	-	0.00	-	0.00	-
Brain	3.50	0.29	4.35	0.35	0.16	0.10	-	-	-	-	0.10	0.01
Spinal Cord	-	-	-	-	-	-	-	-	-	-	0.00	-
Spinal Fluid	-	-	0.00	-	-	-	-	-	-	-	-	-
Blood	-	-	0.10	0.13	-	-	0.00	-	-	-	0.00	-
Muscle	-	-	-	-	0.05	0.10	Trace	-	0.00	-	0.22	0.01
Flat Bone	1.65	10.65	1.95	13.00	2.60	3.25	0.28	1.18	1.03	1.11	18.00	1.02
Long Bone	-	-	0.98	8.00	-	-	-	-	-	-	9.50	1.14
Bone Marrow	-	-	0.18	2.77	-	-	-	-	-	-	-	-
Cartilage	-	-	0.23	0.46	-	-	-	-	0.00	-	0.25	0.26
Lungs	0.25	0.09	1.12	0.08	0.09	0.06	0.09	0.01+	0.00	-	0.08	0.03
Heart	Trace	-	0.58	0.14	0.00	-	0.09	0.03	0.00	-	Trace	-
Thyroid	-	-	-	-	-	-	-	-	0.00	-	-	-
Adrenals	-	-	0.00	-	-	-	0.00	-	0.00	-	-	-
Testicles	-	-	-	-	-	-	Trace	-	-	-	-	-
Pancreas	Trace	-	0.18	0.36	-	-	Trace	-	0.04	0.04	Trace	-
Stomach	-	-	-	-	-	-	0.03	0.01	-	-	-	-
Intestines	-	-	-	-	-	-	0.16	0.01	-	-	0.10	0.02
Bladder	-	-	-	-	-	-	-	-	0.00	-	-	-
Prostate	-	-	-	-	-	-	-	-	0.00	-	-	-
Skin	-	-	-	-	-	-	-	-	-	-	1.93	0.13
Remainder	-	-	-	-	-	-	-	-	-	-	6.00	0.26
Estimated total lead in body	220		1000		240		135		135		36.47	

Normal lead in urine

0.05 mg. - 0.1 mg. lead per 24 hours
0.017 mg. - 0.026 mg. lead/liter

Max. 1.20 mg. lead/liter urine } White lead workers

Normal lead in feces

Average of 3 means page 269, Table 8, Ind. J. Hyg. 15: No. 5, Sept., 1933.
0.0349 mg. lead/gram ash of feces.

0.91 mg. lead/liter urine } Storage battery mfrs.

Transfer to a Squibb separatory funnel.

Shake out with 5 to 10 c.c. of dithizone 1. Discard the dithizone chloroform layer. This extracts tin and bismuth and eliminates the possibility of their appearing later as interfering substances. Lead is not extracted at this hydrogen ion concentration. If it is known that tin and bismuth are not present, the extraction at pH 3.0 to 3.5 may be omitted.

Add 15 c.c. of the citric acid solution.

Neutralize with ammonium hydroxide.

Add 2 c.c. of the potassium cyanide solution.

The material is now ready for lead extraction. In an ammoniacal solution and in the presence of potassium cyanide, dithizone has a selective affinity for lead, forming a cherry-red complex of lead dithizone which is soluble in chloroform.

Allow a small quantity of dithizone to run from a buret into the solution. Shake the solution vigorously. The rapid appearance of a cherry-red chloroform layer indicates the presence of lead. Separate the red chloroform layer and transfer it to another separatory funnel.

Continue the addition of dithizone, the vigorous shaking, and the transferring of the colored chloroform layer until all the lead has been removed. Wash the last traces of lead dithizone through several small portions of chloroform. Collect and save these washings. The proximity of the end point (complete removal of lead) may be roughly estimated by diminution of intensity of the cherry-red color. If it is very red, a larger amount of dithizone may be added. It must be shaken and separated. After each shaking, invert the funnel and open the stopcock to relieve the pressure formed, then close the stopcock.

When all the lead has been extracted, further addition of dithizone will not impart a cherry-red color to the chloroform layer, but upon vigorous shaking of the funnel a green color will form and persist, due to excess of dithizone. The presence of many extraneous salts prevents a sharp end point; that is, a distinct change from red to green. The first titration-extraction is, therefore, not a reliable indication of the quantity of lead present in the specimen.

The end point is often difficult to see because of the lingering transition of colors from cherry-red to pink to violet to blue to green when extraneous salts are present. When this occurs, collect several portions of extracted lead dithizone.

Shake these combined chloroform extracts with 15 c.c. of 1 per cent nitric acid solution. This quantitatively separates the lead into the aqueous solution. Discard the green chloroform layer.

Wash the aqueous layer by shaking with a fresh portion of chloroform to remove any residual dithizone.

Add to the solution several drops of phenolphthalein.

Add ammonia a drop at a time until the solution is alkaline.

Add 2 c.c. of potassium cyanide.

Add citric acid solution drop by drop until the solution is just faintly pink. If the end point is passed, adjust with a drop of dilute ammonium hydroxide.

Titrate the solution with standard dithizone 2. The dithizone may be added in one large portion to within 5 c.c. of the total amount required in the first extraction (when the cherry-red color began to change).

Shake the separatory funnel thoroughly, allow it to stand until the layers separate, and then remove the red chloroform layer.

Continue the titration from this point with great care.

Deliver the dithizone in successive small quantities, approximately 0.5 c.c. each.

Before each shaking add approximately 1 c.c. of chloroform in order that the volume be great enough to enable one to see the color.

Shake vigorously after each addition of dithizone, allow to settle, run out the chloroform layer, and discard it.

Continue in this manner until the end point is reached. This is a persisting light green color in the chloroform layer.

Calculation.—

For standardization, titrate a known quantity of lead solution with dithizone solution using the procedure described above. Since the lead is in pure solution, the preliminary titration-extraction is not necessary. The quantity of lead in the standard used, divided by the number of c.c. of dithizone solution required for the end point, represents the dithizone lead equivalent.

Example.—

The standard contained 20 gammas (0.020 mg.) of lead.

6 c.c. of dithizone were used in the titration.

6 c.c. of dithizone are equivalent to 20 gammas of lead.

20 divided by 6 equals 3.3. Therefore 1 c.c. of dithizone is equivalent to 3.3 gammas of lead.

In the test 21.2 c.c. of dithizone were used.

The specimen, therefore, contains 21.2 × 3.3, or 69.96 gammas of lead per sample analyzed. The aliquot daily factor is then computed.

Summary of Urinary Changes in Lead Poisoning

The urinary changes in lead poisoning are as follows: albumin; granular casts; red cells; abnormal amounts of lead (see above tests); hematoporphyrin.

Summary of Blood Changes in Lead Poisoning

The blood changes in lead poisoning are as follows: low red cell count; low hemoglobin; high blood platelet count; presence of nucleated red cells; changes in the morphology of the red blood cells; basophilia; polychromasia.

(For fuller details, see Hematology of Lead Poisoning in chapter on Hematology.)

Contamination of Samples in Process of Collection and Analysis

Kehoe* especially warns against contaminations of samples intended for chemical analysis for lead for the reason that lead is ubiquitous and reagents of the highest purity show traces of lead. All glassware contains lead and even filter paper and distilled water may have minute amounts of lead in them. Dust suspended in the air of the laboratory may contain significant amounts of lead, especially when there are any painted surfaces. Laboratory surfaces, which have not been proved by chemical test to be lead-free, may contaminate the sample. Necessarily, blanks should be carried along with every set of samwhich have not been proved by chemical test to be lead-free, may contaminate during the collection of samples. Needles and syringes sterilized in the usual manner may introduce lead into the blood or spinal fluid. Bedpans and urinals provide gross contaminations, so that urine samples are likely to be contaminated. Care must be taken to avoid contamination in collecting samples at autopsy. In general, everything that comes in contact with the specimen must be carefully cleaned and selected, and the activities of the patient during the period preceding and during the collection of the sample must be known. A dose of Epsom salts will spoil a fecal sample because of the lead content of the salt employed (Kehoe).

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*Am. J. Clin. Path. 5: No. 1, 1935.

The Quantitative Spectrographic Determination of Lead in Blood and Urine

One of the best and easiest methods of estimating accurately the quantity of lead in blood is by use of the spectrograph. Spectrographic methods of analysis eliminate the annoying and time-consuming measures of avoiding contaminants in reagents, glassware, etc., necessary in the regulation chemical methods. The only possible objection to spectrographic methods in the clinical laboratory is the question of cost of installation. Cholak[1] described his method and views on quantitative spectrographic determination of lead in urine, utilizing a Bausch & Lomb Littrow-type quartz spectrograph. Standard spectrographic methods were used, testing several series of samples containing known quantities of lead in solutions of urine salts equivalent to one-liter samples of urine. The quantities of lead added were such that they fell below the quantity contained in the highest urine standard, that is, 0.20 mg. per liter. He found that concentrations of lead in urine as low as 1 part per 100 million could be detected. For amounts of lead up to 0.10 mg. per liter the accuracy of the method is ± 0.01 mg. per liter. This method is applicable to the measurement of lead in all kinds of biological material.

The method of making the test was the addition of suspected material into the craters of the respective graphite electrodes by the repeated addition from capillary pipettes and the subsequent evaporation of 0.1 c.c. portions. The electrodes were dried in an oven for several hours and then used as the negative poles of the arc. The spectra of the sample and standards are photographed on a 10 × 25 cm. Eastman No. 33 plate. The spectral region taken is that between 2500 and 3400 Angstrom units.

Quantitative spectrographic analysis of lead in blood will probably be the ultimate method of choice in proving lead intoxication. The method of detecting lead in blood by spectrographic analysis may be used with either the grating or the quartz prism spectrograph. Withdraw a given amount of blood, say 5 c.c., from the arm vein, with a chemically clean syringe and needle. Ash; weigh; place in arc of spectrograph. Determine the presence of lead and the quantity of lead.

The Polarographic Method for Detection and Measurement of Lead Absorption

This method was developed through the research of H. A. Cranston, of the Laboratory of Polarographic Analysis, in Chicago, using the Heyrovsky polarograph.[2] He claimed that this new procedure is sufficiently fast and economical for routine periodic examinations, in any plant, of persons subject to lead exposure. This procedure permits accurate interpretation and measurement of current voltage curves by recording all current changes occurring in the course of an analysis.

Technic for the entrainment procedure of Cranston is as follows:

Fill a Pyrex centrifuge tube with fresh urine or with urine that has been preserved with thymol.

Add several reagents in approximate quantities, stirring the solution between each addition. Shake thoroughly.

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[1] J. Am. Chem. Soc. 57: 104, 1935.
[2] Sargent Magazine 1: No. 1, 1941.

Set samples aside.

After precipitation is complete, centrifuge, decant, wash with ammonia water and recentrifuge.

Pour off wash water and allow tubes to drain.

Then add to each tube concentrated hydrochloric acid containing tartaric acid.

Transfer dissolved precipitates to electrolytic cell and wash centrifuge tube with a few drops of distilled water (see Fig. 708).

Add the washings to the solution on the electrolytic cell. A rack which facilitates the complete drainage of the centrifuge tube is shown. Bring to volume with distilled water and note the final volume. See Fig. 709.

Polarographic Measurement of Lead.—Hydrogen, from a tank or any convenient method of generation, is now bubbled through the solution in the electrolytic cells. This may be accomplished easily by the simple rubber stopper and tube arrangement illustrated. See Fig. 710.

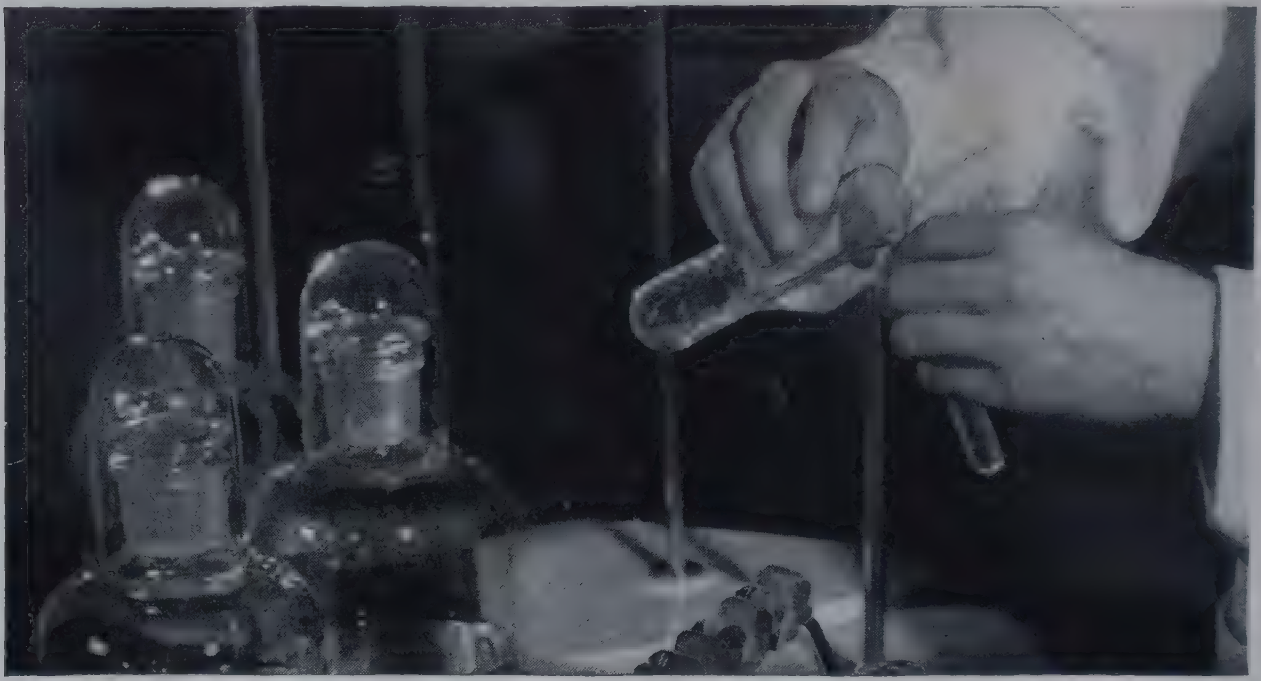

Fig. 708.—Transferring precipitate to electrolytic cell for polarographic determination of lead.[2]

Remove the stoppers and insert the dropping mercury electrode into the tube so that it is below the surface of the liquid. Connection is made through a suitable mercury pool with the lower terminal of the cell.

Adjust polarograph. The power switch of the polarograph is turned on, the potentiometer drum turned to about minus 0.2 volt, the galvanometer beam adjusted to the zero end of the deflection scale, the shutter opened and the motor started. The recording is continued to a bridge reading about minus 0.6 volt, requiring a run of about 2 to 3 minutes. The motor is then stopped, the shutter closed, and the camera drum rotated to a starting position for the next sample record. The capillary tube is shifted to the next sample tube and the operations are repeated. Ten records may be made on a single sheet without reloading the camera. See Fig. 711.

To reload, the complete drum or camera is removed from the polarograph.

Typical lead curves. After development of the photographic paper the curves are measured and compared with a similar curve run occasionally with a standard solution. See Fig. 712.

Calculation of Urinary Lead.—No attempt is made here to discuss the theoretical aspects of polarographic analysis. It will suffice for the present purpose to state that the

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[2] Figs. 708 to 713 courtesy Sargent Magazine **1**: No. 1, 1941.

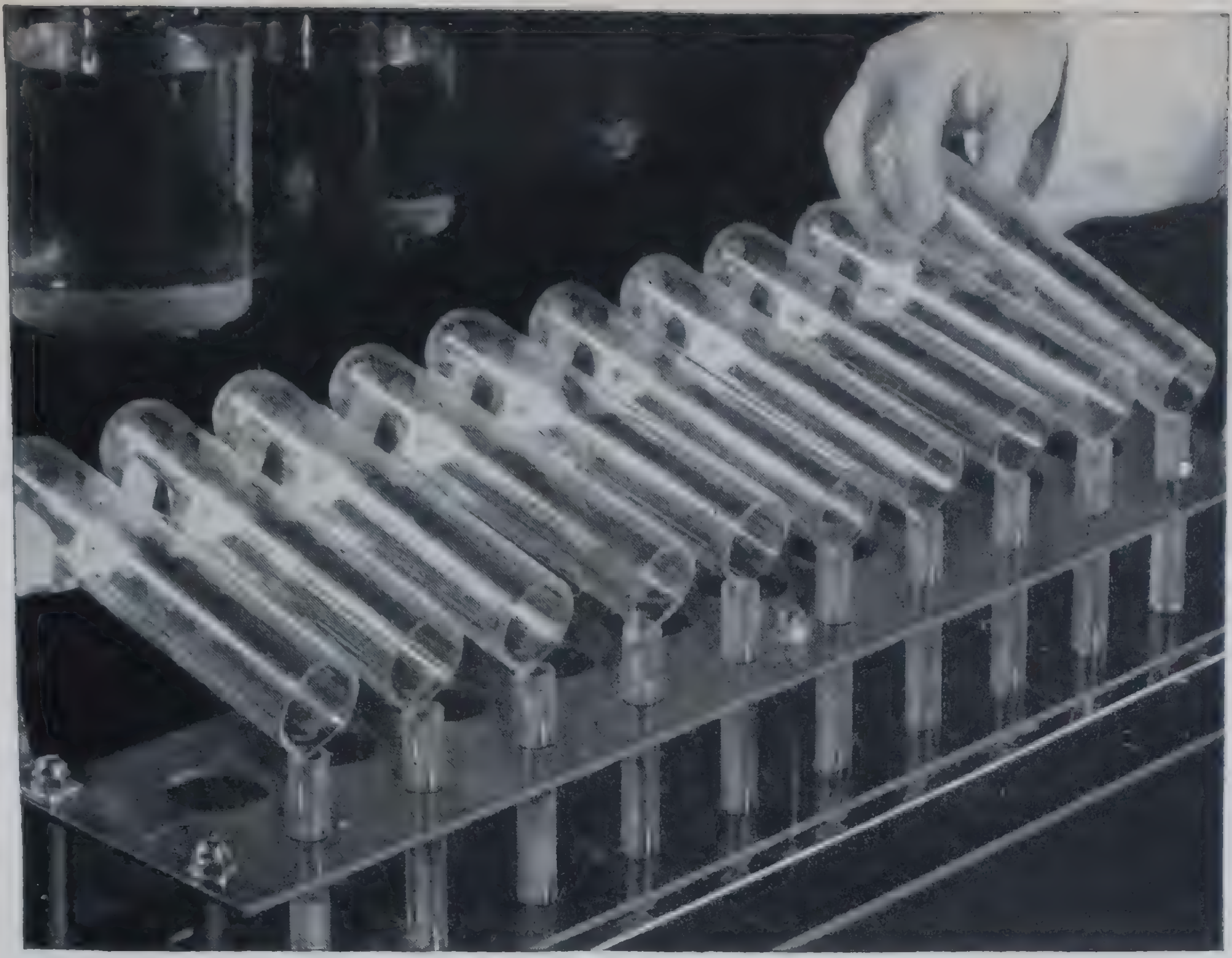

Fig. 709.—Rack to facilitate complete drainage of centrifuge tubes.

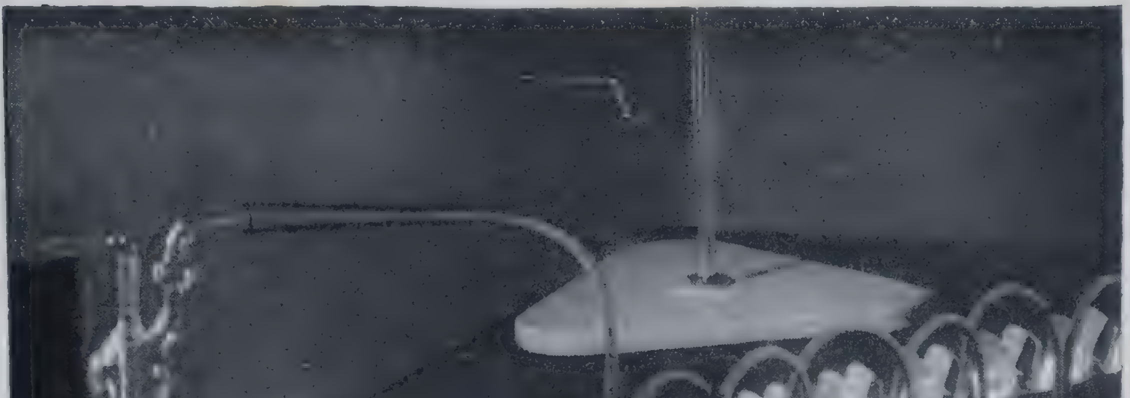

Fig. 710.—Bubbling hydrogen through solution in electrolytic cells.

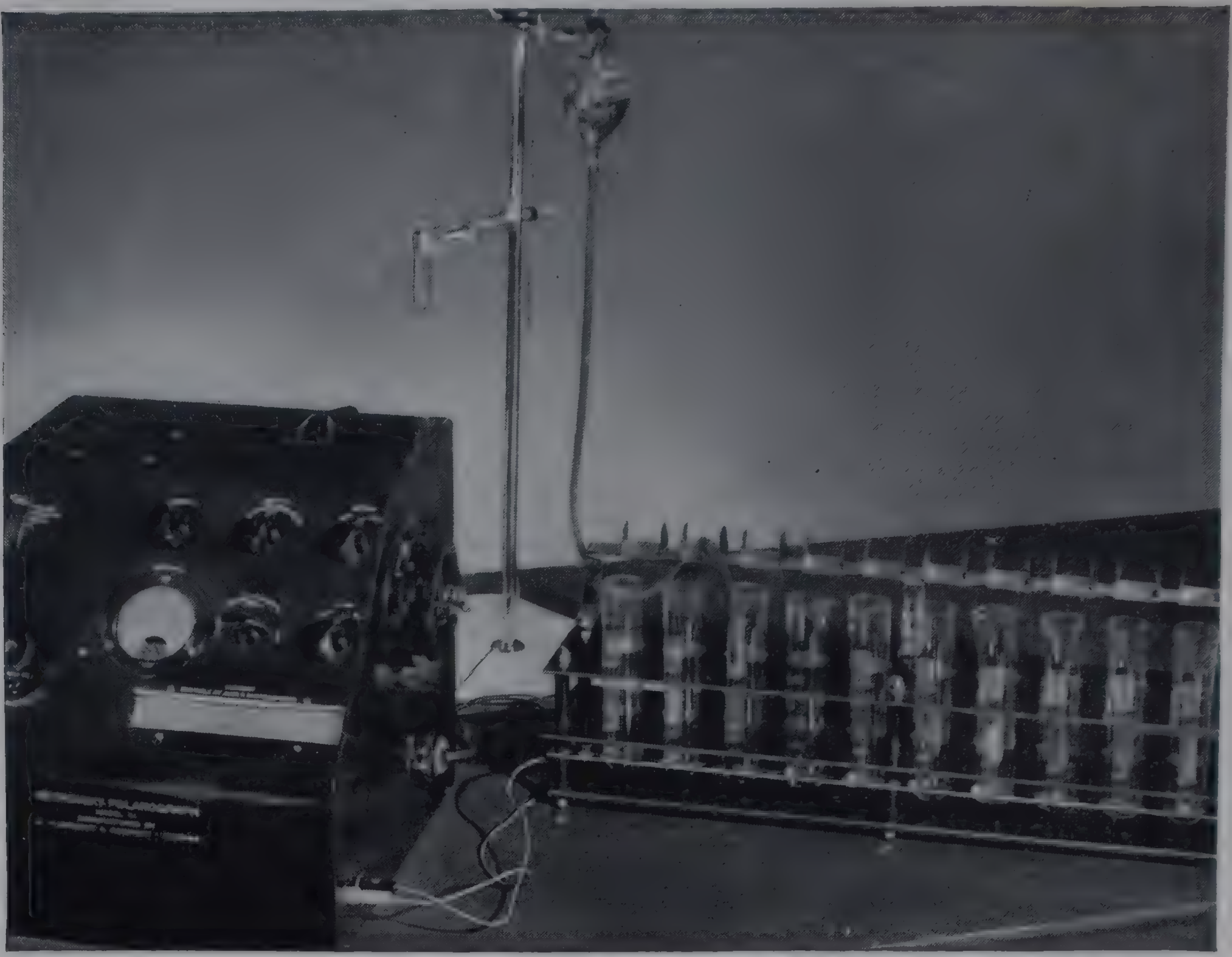

Fig. 711.—Adjusting polarograph.

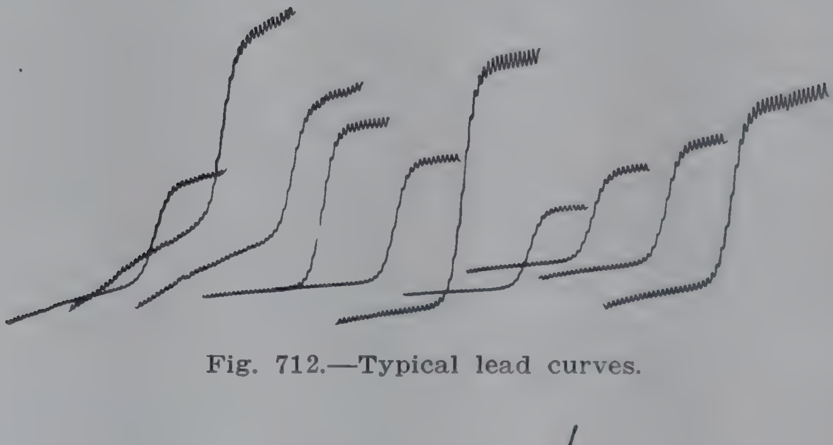

Fig. 712.—Typical lead curves.

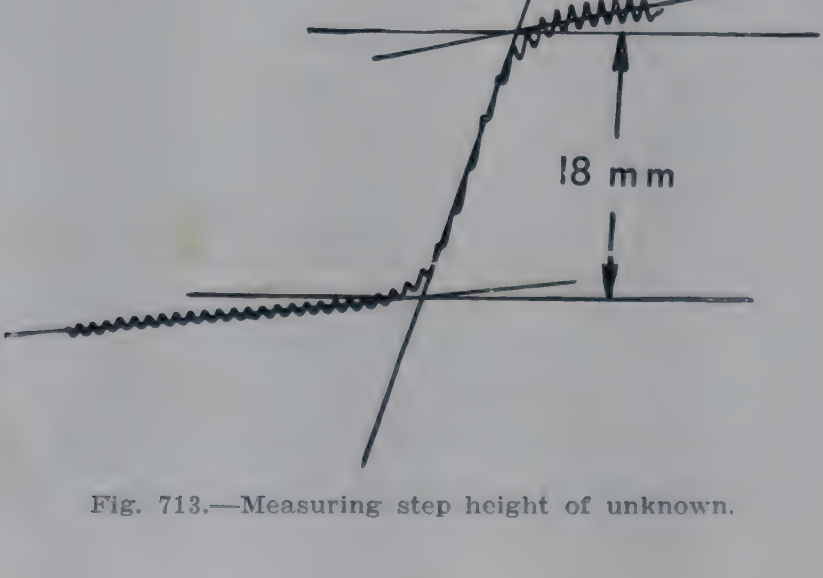

Fig. 713.—Measuring step height of unknown.

height of the step for each ion present in a solution is directly proportional to the concentration of that ion. Therefore, it is only necessary to measure the step height by the method shown, and compare it with the height of a standard solution. See Fig. 713.

Example:

Effective concentration of standard	10	micrograms/c.c.
Step height of standard	45	mm.
Step height of unknown	18	mm.
Volume of unknown	2.0	c.c.

Therefore, the concentration of the unknown is $\frac{18 \times 10}{45} =$ micrograms/c.c.; and as 2 c.c. contained the lead originally present in 80 c.c. of urine, the amount of lead present in 1 liter of urine will be $\frac{1,000}{80} \times 2 \times 4$, or 100 micrograms/liter.

Normal Urinary Lead Values

Many determinations of lead in urine have been made on individuals with no known history of exposure to lead. Gettler and Kaye found that the lead content in normal individuals varies widely. In all cases, however, the concentration was below 100 gammas* in a 24-hour specimen. Other workers have corroborated these findings.

RADIOACTIVE SUBSTANCES

Radium and Uranium

In testing for radium and uranium the bones and teeth are the tissues of choice. Gettler found that they contain more of the accumulated radium than do the soft tissues.

Ash a definite weight of bone. Test the ash for radioactivity by:

1. the Geiger-Müller counter;
2. the electroscope;
3. exposing an x-ray plate.

Place the bones or the ash on an unexposed x-ray plate which has been covered with black paper.

Allow this to stand in a dark room for a week or more.

Develop the plate.

If radioactive material was present in the material tested an exposed light space will appear on the plate in the area on which the specimen was placed. Both beta and gamma rays of radioactive substances will penetrate the black protective paper on the film and will bombard and expose the area of contact.

UNCOMMON OCCUPATIONAL METALLIC POISONS

For the detection of the more uncommon occupational metallic poisons which may be present in fair amounts in specimens submitted for analysis, reference should be made to a standard text on qualitative and quantitative analysis. A satisfactory scheme is found in Noyes textbook on qualitative and quantitative analysis. Any similar scheme may be used.

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*0.001 milligram = 1 gamma; 100 gammas = 0.1 mg.

Group I.—

Concentrated hydrochloric acid precipitates silver (w),* lead (w), mercury (w).

Group II.—

Hydrochloric acid, hydrogen sulphide precipitates lead (bk), bismuth (bk), copper (bk), cadmium (y), arsenic (y), mercury (bk), antimony (o), tin (y).

Group III.—

Ammonia, hydrogen sulphide precipitates iron (bk), aluminum (w), chromium (gn), cobalt (bk), nickel (bk), manganese (p), zinc (w).

Group IV.—

Ammonium carbonate hydroxide reagent precipitates barium (w), strontium (w), calcium (w), magnesium (w).

To Generate Hydrogen Sulphide

Use a large test tube into which is placed a one-hole rubber stopper. One end of a piece of glass tubing, bent in a ''U'' shape, is placed through the hole of the rubber stopper so that it extends just within the test tube.

To generate hydrogen sulphide, place a small amount of sulphide of iron in the test tube and add a few c.c. of dilute hydrochloric or sulphuric acid, and tightly stopper with the rubber stopper and glass tubing. The gas which is generated passes through the glass tubing into the solution to be tested. Or use the Kipp generator (Fig. 715).

GROUP V

IDENTIFICATION OF GASES OF TOXIC NATURE IN BLOOD

Detection of Carbon Monoxide

Poisoning by a small amount of carbon monoxide may produce very few changes, or if the patient lives for a number of hours after exposure, only a careful examination of the blood will reveal the presence of the gas. Where the blood is well saturated with the gas, the surface of the body is cherry red. There are frequently red patches upon the thighs and the front of the trunk and neck. The blood is rather characteristic, being bright cherry red, usually fluid, and coagulating slowly. It should be placed in a container which is sealed immediately after collection.

Detection of carbon monoxide may be carried out by means of the spectroscope, as outlined on page 2359. Shake diluted blood with from 100 to 200 c.c. of air and examine by the spectroscope. Blood taken from persons or animals poisoned by carbon monoxide shows a characteristic absorption spectrum closely resembling that of oxyhemoglobin. The absorption occurs between the D and E lines. There are two absorption bands of nearly equal intensity and width. The one near D coincides almost exactly with that of oxyhemoglobin of the same position; the space between it and the band toward F is considerably wider than that between the two bands of the oxyhemoglobin spectrum. In making the test, the ordinary spectroscope may be used. The blood contained in the test tube is placed in the path of the rays from the lamp illuminating the spectroscope. Dilute the blood in making the test.

Other tests for carbon monoxide are as follows:

Bloods rich in carbon monoxide, when boiled, yield a brick-red mass; ordinary blood becomes brown black.

Ten c.c. of 2% solution of a blood strongly charged with carbon monoxide, with 2 c.c. of yellow ammonium sulphide, and 0.2 c.c. of 30% acetic acid, yields a bright red, while normal blood yields a green precipitate.

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*(w) white; (y) yellow; (o) orange; (bk) black; (gn) green; (p) pink.

When blood is added to dilute hydrogen sulphide water drop by drop, it produces a bright red precipitate if carbon monoxide is present; otherwise it is bright green.

Normal blood when shaken with one or two volumes of a solution of sodium hydroxide of 1.3 specific gravity becomes black, in thin layers dark greenish brown; carbon monoxide blood remains red and appears like red lead.

Two c.c. of carbon monoxide blood with an equal quantity of water and 3 drops of a one-third saturated solution of copper sulphate when mixed produce a brick-red precipitate, while normal blood yields a greenish brown one.

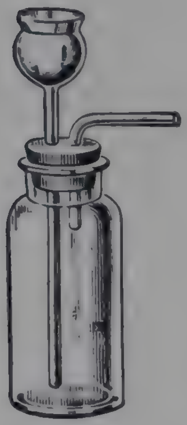

Fig. 714.

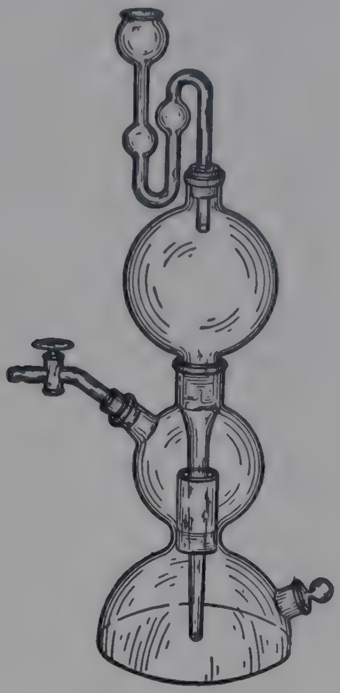

Fig. 715.

Fig. 714.—Gas generating bottle. (Courtesy Chemical Rubber Company.)
Fig. 715.—Kipp gas generator. (Courtesy Chemical Rubber Company.)

Quantitative Determination of Carbon Monoxide

The method of Fodor[1] is as follows:

The method of Fodor, based on the reduction of palladous chloride by carbon monoxide, is much better for the examination of carbon monoxide in blood. The reaction is expressed by the equation: $PdCl_2 + CO = Pd + 2HCl + CO_2$. For the examination of the blood, dilute 5 c.c. with 20 c.c. of water in a large test tube of 80 c.c. capacity; add a small piece of sodium hydroxide to aid in the expulsion of carbon monoxide. A tube containing 10 c.c. of palladous chloride precedes the tube containing the blood to remove the carbon monoxide from the laboratory air. A third U tube following the tube containing the blood contains a solution of lead acetate, and a fourth tube contains dilute sulphuric acid. The chain of tubes is completed by the addition of three U tubes containing a solution of palladous chloride.[2] Heat test tube containing the blood in a water bath to 90° C., while a gentle current of air, previously drawn through the tube of palladous chloride, is conducted through the chain of tubes at the rate of 300 c.c. per hour for four hours. If carbon monoxide is present, the palladous chloride is reduced, and a black precipitate of palladium will be deposited. Collect reduced palladium on a filter paper, wash with water, and dissolve in hot nitrohydrochloric acid. The U tubes, after being washed with water, will have a small amount of palladium adhering to the sides.

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[1] Deutsch. Vrtljschr. f. off. Gesundhtspfl. 12: 377, 1880.

[2] The commercial salt is dissolved in hydrochloric acid, with the addition of nitric acid, evaporated to dryness on the water bath, moistened with hydrochloric acid, dried, taken up with a small amount of water and again dried to free it from excess acid. The residue is then made up to volume so that 500 c.c. will contain 1 gm.

This can be dissolved in the hot nitrohydrochloric acid and added to that dissolved from the filter paper. Evaporate the solution on the hot water bath, take up with strong hydrochloric acid three or four times to remove all traces of nitric acid. Dissolve the residue in water and titrate with a solution of potassium iodide containing 1.486 gm. to the liter. Heat the diluted palladous chloride solution on the water bath before each addition of the potassium iodide solution. Filter off a brown precipitate of palladous iodide and repeat the operation until the addition of potassium iodide fails to produce a brown cloudiness. Each cubic centimeter of the potassium iodide solution represents 0.1 c.c. of carbon monoxide. In case the blood contains only a small amount of carbon monoxide, as is demonstrated by the tannic acid and other color tests, 20 c.c. of the blood are used in a small Erlenmeyer flask. When it is desired to report the saturation of the blood after obtaining the percentage of carbon monoxide by volume, one can use the relation x : 20, since a saturated blood will hold in combination 20% by volume of carbon monoxide.[3]

A Simple Method for Carbon Monoxide in Blood

Method of Gettler and Freimuth[4]

This method was devised to serve as a simple and rapid procedure for the routine analysis of carbon monoxide in blood. The determination can be completed in 15 minutes, excluding the preparation of standards, and requires no personal attention during the test.

Principle.—

The carbon monoxide is liberated from carboxyhemoglobin by potassium ferricyanide, and by aeration it is passed through a disk of palladium chloride paper. The carbon monoxide produces a dark stain on the filter paper, due to the reduction of palladium ions to metallic palladium according to the reaction $CO + PdCl_2 + H_2O \rightarrow Pd + CO_2 + 2HCl$. The depth of stain is proportional to the amount of carbon monoxide present.

Apparatus (See Fig. 716).—

3 potato tubes, 5 to 6 inches long, ¾ inch wide
1 glass flange (see Fig. 572)
1 2-liter Marriott flask, or a larger flask
2 screw clamps
Glass beads

Reagents.—

Palladium Chloride Solution.—

Dissolve 0.5 gm. palladium chloride
in 0.5 c.c. concentrated hydrochloric acid and
dilute to 50 c.c. with distilled water.

Ferricyanide Solution.—

Dissolve 3.2 gm. potassium ferricyanide ($K_3Fe(CN)_6$)
and 0.8 gm. saponin in distilled water and dilute to
100.0 c.c. with distilled water.

Lactic Acid Solution.—

Place 0.8 c.c. lactic acid, sp. gr. 1.20, in distilled water in a 100 c.c. volumetric flask, and dilute to 100 c.c. with distilled water. Mix well.

Ferricyanide-Lactic Acid Solution.—

Mix equal parts of each.

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[3] See Florentin and van den Bergh: Compt. rend. Acad. d. sc. **172**: 391, 1921; Nicloux, M.: Bull. soc. chem. biol. 2: 171, 1902; Micromethod for Estimating Carbon Monoxide, Ibid. 3: 286, 1921.

[4] Am. J. Clin. Path. **13**: 9, Sept., 1934.

Lead Acetate.—

Dissolve 10 gm. lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) in distilled water in a 100 c.c. volumetric flask, and dilute to 100 c.c. with distilled water.

Caprylic Alcohol.—

Technic.—

Tube "A."—Place 5 c.c. of palladium chloride solution in tube "A."

Tube "B."—

Place 2 c.c., accurately measured, of the blood to be analyzed, in tube "B."
Add 4 c.c. of the ferricyanide solution
and 2 drops of caprylic alcohol.

Tube "C."—

Place a 1½ -inch layer of glass beads in tube "C."
Add 5 c.c. of the lead acetate solution.
Set up the apparatus as indicated in Fig. 716.

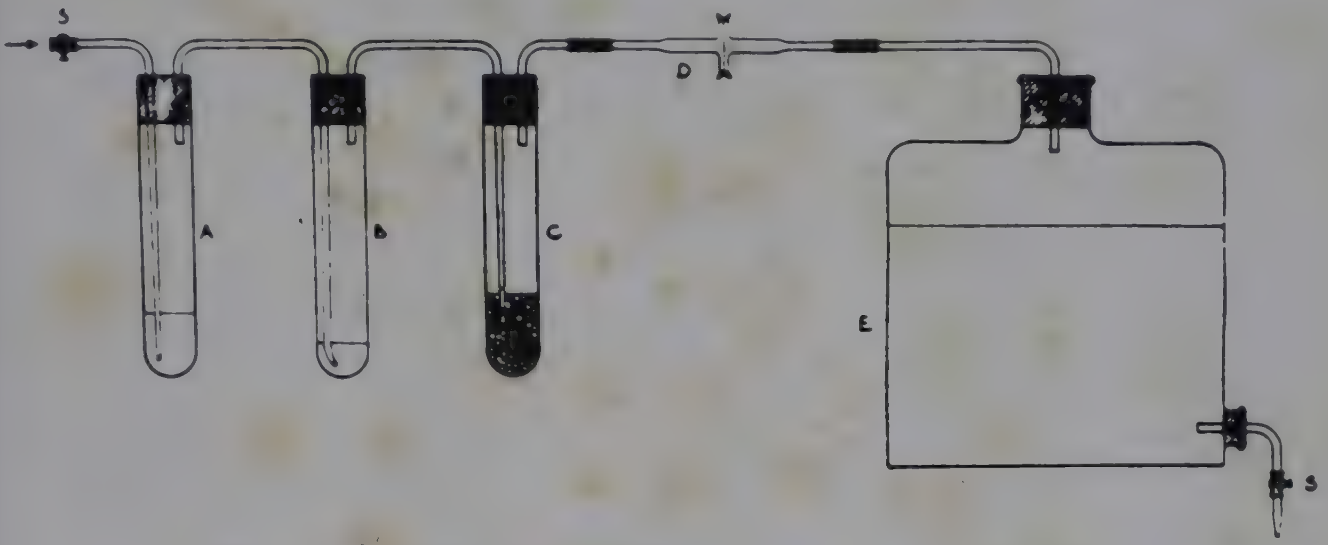

Fig. 716.—Apparatus for estimating carbon monoxide in blood.

Place between the two glass flanges "D" a moist disk of *palladium paper* prepared as follows:

Palladium Paper.—

Cut, from a piece of Whatman No. 3 filter paper, a disk just slightly smaller in circumference than the ground glass surface of the flange. Then moisten the disk with a few drops of palladium chloride solution. Any excess solution must be removed by pressing the disk between two pieces of blotting paper. Then place the moist disk between the flanges and fasten together by means of rubber bands. They must be fastened tightly to prevent leakage at this part of the apparatus.

The *Marriott flask* "E" contains a large volume of tap water.

Begin the aeration by slightly releasing the screw clamp "S" on the rubber tube connection of the outlet tube of bottle "E," which allows water to flow out slowly. So regulate the rate of flow, with either one of the screw clamps "S," that 400 c.c. of water will run out in about 15 minutes. As a result of the removal of this amount of water, 400 c.c. of air will have been slowly drawn through the entire apparatus. Air is drawn first through the palladium chloride solution "A" to remove from it any carbon monoxide which might be present. This washed air then passes through the blood in "B" and carries any carbon monoxide present in the blood along with it. The stream of air containing the carbon monoxide next passes through the lead acetate solution in "C."

Here any hydrogen sulphide that may be present in the blood due to putrefaction is removed, and the carbon monoxide passes through unaffected. The stream of air containing the carbon monoxide passes along and is carried through the flanges where it meets the disk of moist palladium chloride paper. The carbon monoxide reduces the palladium ions to metallic palladium, and as a result a dark circular stain is produced. The depth of stain is proportional to the amount of carbon monoxide present.

Discontinue aeration after 15 minutes.

Remove the disk of palladium chloride paper from the flange, and wash it thoroughly with distilled water to remove all unchanged palladium chloride. Then compare the stain with a series of standard stains in order to estimate the per cent of carbon monoxide saturation of the blood.

Standard Stains.—

Prepare standard stains in a similar manner by saturating normal blood with carbon monoxide gas for about 15 minutes. Then assuming this to be 100% saturation, make various dilutions of this blood with other normal blood samples to produce the desired per cent of saturation. For example, a mixture of 1 part of 100 per cent saturated blood and 1 part of normal blood will produce 50 per cent carbon monoxide saturation. Lower carbon monoxide-containing samples may be prepared by mixing the 50 per cent carbon monoxide blood with normal blood.

After preparing, washing, and drying these standard stains, mount them on a cardboard. Keep them away from fumes, heat, or direct sunlight, and they will remain in good condition for several months.

The carbon monoxide content of the blood of patients who have been removed from a carbon monoxide atmosphere and brought to a hospital has never been found to be higher than 50 per cent saturation.

It is evident that an approximate estimation only may be made by this method. For more accuracy a correction must be made. Determine the per cent of hemoglobin in the blood and correct as follows:

$$\frac{100}{\%\text{ of hemoglobin}} \times \text{carbonyl hemoglobin saturation} = \begin{array}{c}\text{per cent saturation with}\\ \text{carbon monoxide.}\end{array}$$

Example.—

If the per cent of hemoglobin is 90 and the carbonyl hemoglobin saturation is 20, then $\frac{100}{90} \times 20 =$ per cent saturation with carbon monoxide.

For a more accurate quantitative determination of carbon monoxide, the manometric technic of van Slyke should be used.

For a rapid qualitative indication and semi-quantitative estimation of carbon monoxide, use the following methods. These tests are not sensitive, however, below 15 per cent carbonyl hemoglobin saturation.

Dilution Test.—

Add 1 drop of blood to 10 c.c. of distilled water. Compare with normal blood treated in a similar manner. Blood containing carbon monoxide will be cherry-red while the normal blood will be brownish red.

Alkali Test.—

Add several drops of 10% sodium hydroxide solution to blood diluted 1 drop to 10 c.c. of water. Blood containing carbon monoxide remains a cherry-red whereas normal blood turns a yellow-green.

Welzel Test.—

Add a solution of tannic acid to the diluted blood. A brick-red precipitate forms in the presence of carbon monoxide.

Sayer-Yant Pyrotannic Acid Test.—

Dilute 1 c.c. of blood with 9 c.c. of distilled water.

Add 10 c.c. of pyrotannic acid solution.

Stopper the tube, shake, allow to stand 15 minutes.

Normal blood is gray-brown; blood containing carbon monoxide retains a pink color.

Pyrotannic acid solution is made by dissolving 1 gm. of pyrogallic acid and 1 gram of tannic acid in 100 c.c. of distilled water.

Note.—Both the Welzel test and the Sayer-Yant test may be used as a rough estimation of the quantity of carbon monoxide present, since the color intensities produced are proportional to the concentration of carbon monoxide.

Gettler and Freimuth reported carbonyl hemoglobin saturation of blood of 30 to 90 per cent in deaths due to carbon monoxide poisoning.

Postmortem absorption of carbon monoxide is very small; the quantity absorbed after death is practically negligible, less than 2 per cent.

Carbon monoxide is not produced by putrefaction.

Embalming fluid interferes with CO determination.

It seems reasonable to assume, therefore, that carbon monoxide found in the blood was absorbed during life.

Hydrogen Sulphide

Hydrogen sulphide may be isolated by steam distillation. Distill over steam *fresh* organs or body fluids. During this distillation the very characteristic hydrogen sulphide odor of rotten egg is detectable, if hydrogen sulphide is present.

Add a small aliquot of the distillate to a solution of lead acetate. If a black precipitate of lead sulphide forms, hydrogen sulphide was present in the original specimen.

A spectroscopic examination for sulfhemoglobin may be made. This spectroscopic test is unreliable if the individual has recently been treated with sulfa drugs.

Cyanide in Blood and Tissues

Micromethod of Gettler and Goldbaum

The micromethod of Gettler and Goldbaum for detection of cyanides in blood and tissue combines the simplicity of a spot test with the specific Prussian blue reaction. By the use of an impregnated paper and a special flange arrangement, it is possible to detect micro quantities of cyanide and to estimate the concentration by comparison with spots similarly prepared from cyanide solutions of known concentration.

Ferrous Sulphate Impregnated Paper.—

Dissolve 10 gm. hydrated ferrous sulphate in 100 c.c. of distilled water. Remove insoluble material by filtration.

Pour the clear solution into a large Petri dish.

Immerse Whatman No. 50 filter papers in the ferrous sulphate solution for 2 to 5 minutes. Remove and suspend in the air to dry.

Place 20% solution of sodium hydroxide in another Petri dish.

Dip the dry ferrous sulphate papers in this solution for a few seconds, remove, and suspend in the air until they are only slightly moist.

Prepare fresh papers on the day of the analysis.

Flanges.—

Use the flanges made by Eck and Krebs of New York. See Fig. 691. These are of varying diameters, 8 mm., 4 mm. Place the moist paper disk in the flange and close by means of a rubber band.

20% Trichloroacetic Acid.—

20 gm. trichloroacetic acid dissolved in distilled water and diluted to 100 c.c. with distilled water.

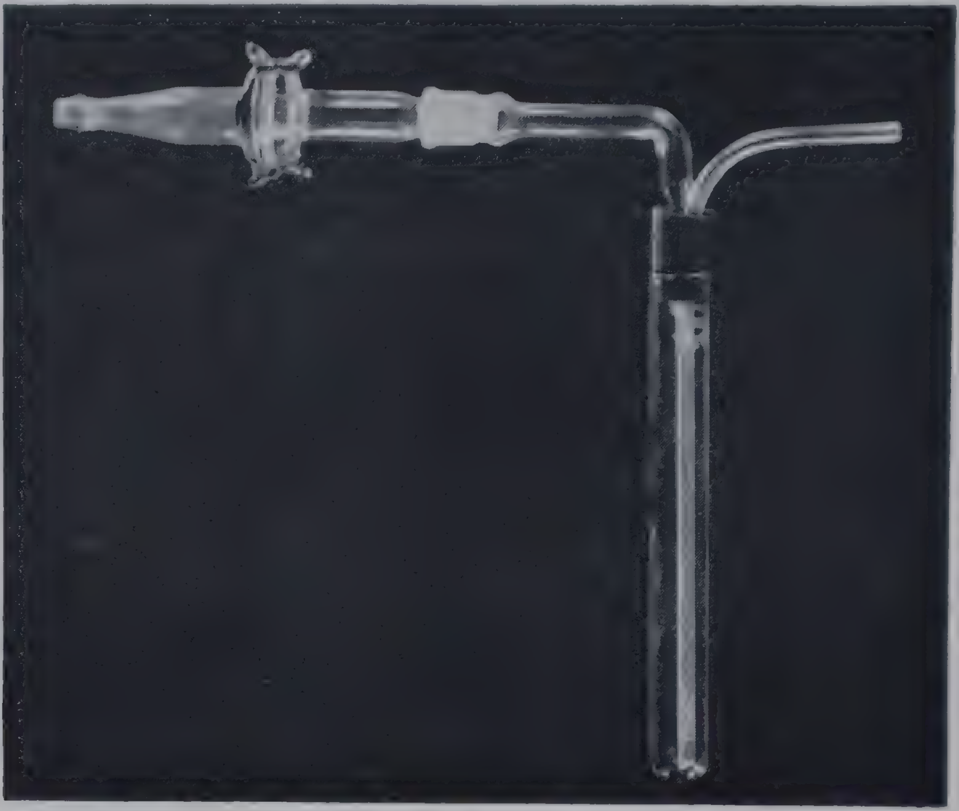

Fig. 717.—Apparatus for the Gettler-Goldbaum spot-test for cyanide.

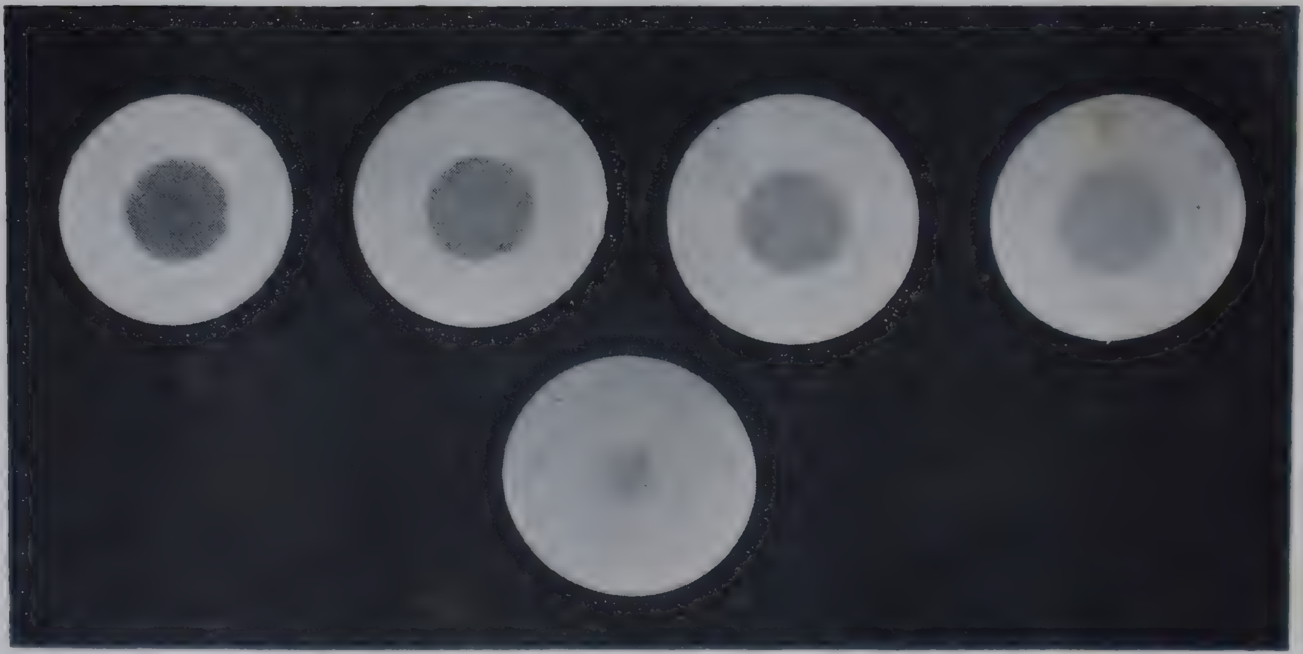

Fig. 718.—Gettler-Goldbaum cyanide spot-test. Standards containing 5, 4, 3, 2, and 1 microgram of cyanide.

Technic.—

Transfer 1 c.c. of blood or 1 gm. of tissue to a 20 c.c. test tube.

Assemble the apparatus as shown in Fig. 717 with the moistened paper disk in place.

Place the test tube in a boiling water bath and apply suction gently while running 2 c.c. of 20% trichloroacetic acid through the inlet tube into the test tube. The trichloroacetic acid helps prevent foaming by precipitating the proteins. It also helps liberate hydrogen cyanide.

Now apply suction at full force.

If water droplets condense around the joint of the apparatus, turn the suction down to keep the water from wetting the paper.

Stop the suction at the end of 5 minutes.

Remove the paper and dip it into a small beaker containing warm 1:4 hydrochloric acid. An insoluble precipitate of Prussian blue develops in the center of the paper and soluble iron salts are formed if cyanide is present. The iron salts may be removed by washing the paper with water. This leaves the insoluble Prussian blue impregnated filter paper.

To make a quantitative determination, compare the blue color with spots prepared from cyanide solutions of known concentrations treated in a similar manner. See Fig. 718.

With the 8 mm. flange, concentrations of cyanide as low as 1 microgram can be detected. Quantities of 1 to 5 micrograms can be estimated within 0.5 microgram by comparison with a series of standards containing, respectively, 1,2,3,4, and 5 micrograms of cyanide.

Sensitivity may be increased to detect concentrations of 0.2 microgram of cyanide by using 4 mm. flanges.

The Spectroscope

Spectroscopic Tests for Carbon Monoxide and Other Substances.—

The discovery of Hoppe-Seyler in 1862 of the spectrum of oxyhemoglobin led to the introduction of the spectroscope into forensic medical practice. Later the spectrum of carbon monoxide hemoglobin was worked out and its irreducible character demonstrated so satisfactorily that no examination for this substance is complete without a spectroscopic control. (See pages 2352 ff. for detailed examination for carbon monoxide.)

A prism is a triangular piece of transparent glass. When light is projected on a prism, two results are brought about.

1. The rays of light are divided from the plane and an **angle of deviation** is formed.

2. The rays of light are divided into their component parts, giving a **band of colors** ranging from red to violet. This phenomenon is called **dispersion.** When this band of colors is focused upon a screen, a **spectrum** is formed.

The **spectroscope** is an instrument used to view the spectrum. There are two general types of spectroscope; namely, the direct vision, and the single prism.

Description of the Instrument

The **Direct Vision** spectroscope consists of a cylindrical tube provided with an ocular, and containing a prism, a draw tube with adjustable slit, and a telescope or collimator tube with a convex lens to render the rays parallel. At the end of the collimator is a slit. When a beam of light enters through the slit, it is dispersed and a band of colors can be seen through the instrument.

The **Simple Prism** spectroscope consists of a prism and two telescopes directed toward the prism. The light is allowed to enter one tube, or telescope, called a collimator, through a narrow slit at its distal end, while at the other end a convex lens serves to collect the light into nearly parallel beams. These rays pass through the prism where dispersion and deviation are brought about. The spectrum is observed through a second telescope.

The second telescope is equipped with a simple microscopic device. There is in the instrument a third telescope equipped with a scale for measurements of light at different points on the spectrum. The measurements are in microns. The prism is located in the instrument in such manner that it will rotate at different points on the scale. The scale is photographed above the spectrum, the divisions being placed so that the measurement of bands of light can be recorded. A direct reading of bright line spectra or absorption bands can be made. (See below.)

A luminous Bunsen flame, or an electric light serves as the source of illumination.

Principle

When a luminous flame of a candle, oil, or gas is examined spectroscopically, it shows **a continuous spectrum**; that is, this white light has been decomposed into its component parts.

If a metal or its salt is burned on a nonluminous flame, the spectrum is not continuous, but, instead, bright lines, close together, will appear at different colors of the spectrum. These lines are called **bright line spectra**. For example, if a platinum wire is dipped into sodium chloride, and placed in a flame, and this light allowed to shine through the spectroscope, two bright lines very close together in the yellow portion of the spectrum are seen. Since this spectrum is specific, sodium can be identified by it. The other colors are very dim. All other metals and their salts, such as, potassium, lithium, calcium, strontium, can be identified by spectroscopic examination. These spectra can be seen only when the substance is heated to a point where volatilization takes place, because gases alone show the property of giving **discontinuous**, or **bright line**, spectra. **No two gases give the same spectra**. In setting up the instrument, the scale is put in position by means of the spectra from sodium and strontium. Luminous flames give a continuous spectrum because the luminosity is due to light emitted by particles of **solid** carbon floating in the burning gas.

If it can be so arranged that a continuous spectrum is produced from a strongly luminous flame, and a gaseous compound placed between the light and the slit in the spectroscope, there is no longer a continuous spectrum. A **black line** intercepts it. When portions of light are absorbed by vapors, in the manner just described, the spectrum is **an absorption spectrum**. When light passes through solutions of blood, light absorption spectra are produced.

In studying different solutions of blood, the specimen is diluted so that the density is not very great. A portion of diluted specimen is placed in a glass absorption cell which has a width of about 2 to 3 mm. The cell containing the specimen is held in place in front of the source of light. The source of light is a very strong electric light. There will be formed different absorption bands (dark bands) for different substances in the blood specimen; such as, hemoglobin, oxyhemoglobin, methemoglobin, carbon monoxide, cyanide, etc.

Examples

Bright Line Spectra

Sodium	0.5896 and 0.5893.
Potassium	0.7699 and 0.7666.
Lithium	0.6708
Hydrogen	0.6563 and 0.4867 and 0.4308
Thallium	0.5351
Magnesium	0.5184 and 0.5173
Strontium	0.4608

Absorption Spectra

Hemoglobin	0.5896 to 0.5305 and 0.8100 to 0.76 and 0.451 to 0.456.
Oxyhemoglobin (very weak dilution)	0.84 to 0.815 and 0.583 to 0.575 and 0.5525 to 0.53 and 0.4225 to 0.4215.
Methemoglobin	0.72 to 0.70 and 0.641 to 0.635 and 0.62 to 0.6125 and 0.595 to 0.5875.
Carbon Monoxide Hemoglobin	0.72 to 0.6975 and 0.5825 to 0.5625 and 0.549 to 0.5289 and 0.4425 to 0.4375.
Acid Hematin	0.7125 to 0.70 and 0.6525 to 0.62 and 0.56 to 0.5267.

Acid Hematin in alcoholic solution,	0.7175 to 0.70 and 0.6533 to 0.62 and 0.588 to 0.58 and 0.555 to 0.5267 and 0.51 to 0.496 and 0.46 to 0.4533.
Acid Hematoporphyrin	0.7175 to 0.73 and 0.61 to 0.586 and 0.562 to 0.5575 and 0.54 to 0.536 and 0.4575 to 0.45.
Alkaline Hematoporphyrin	0.722 to 0.7067 and 0.6345 to 0.61 and 0.572 to 0.56 and 0.542 to 0.53 and 0.515 to 0.51 and 0.4925 to 0.4875 and 0.449 to 0.445.
Hemachromogen	0.825 to 0.80 and 0.57 to 0.5633 and 0.555 to 0.55 and 0.535 to 0.515 and 0.469 to 0.466.

Cyanide—The blood should be diluted 1:100. Normal blood is also diluted 1:100. To both specimens, add some neutral hydrogen peroxide, taking care to avoid an excess. The normal blood retains its red color and its oxyhemoglobin absorption bands. The cyanide blood changes in color from red to brownish red, brown, yellow, and finally white. Spectroscopically, during these color changes, the absorption bands of the oxyhemoglobin are changed into those of methemoglobin, and these finally disappear, leaving no absorption bands at all.

GROUP VI. MISCELLANEOUS METHODS

FLUORIDES

Fluoride compounds have been used in various insecticides. Fluoride poisoning is not too uncommon an occurrence. Symptoms are similar to the general symptoms of acute metallic poisoning—cramps, abdominal pain, purging, etc.

Etch Test

Using sodium hydroxide solution, alkalinize a small aliquot of either tissue or body fluid.

Ash at low heat.

Transfer the ash to a small *lead* dish.

Coat a small glass plate with paraffin. Carefully scratch several small characteristic markings through the paraffin in such manner that the glass surface is exposed through the scratches.

Add several c.c. of concentrated sulphuric acid to the contents of the lead dish in order to liberate hydrofluoric acid.

Quickly place the glass plate, paraffin side down, over the opening of the lead dish so that the entire dish is covered.

Leave the glass plate in this position for 30 to 60 minutes.

If the specimen contains fluorides, the exposed glass will be etched.

When the reaction is complete, remove the slide or glass plate, wash off the paraffin by means of an organic solvent, and observe the exposed areas for etching. They should correspond to the characteristic markings originally scratched through the paraffin.

To increase the sensitivity of this test, use a lead cover with an opening small enough to allow only a small area in the dish to liberate the hydrofluoric acid. Place the slide over this area.

This test is specific for fluorides.

Gettler Test

Alkalinize 1 to 3 c.c. of stomach contents or urine with several drops of sodium hydroxide solution.

Dry at low heat in a small glass container or porcelain crucible.

To the dry residue add a small amount of powdered glass and mix thoroughly.

Acidify the mixture with several c.c. of concentrated sulphuric acid.

Cover immediately with a small glass plate from the under surface of which is hanging a drop of 5 per cent sodium chloride.

Heat the crucible gently. To prevent evaporation of the drop of sodium chloride hanging down from the under surface of the slide, place a small piece of ice, or a small container of ice, on top of the slide.

After 3 to 5 minutes, carefully remove the slide and examine the drop.

If fluorides are present in the specimen, silicon tetrafluoride is formed. This very volatile substance rises and combines with the sodium chloride drop to form typical crystals of sodium fluosilicate.

Examine under the microscope. The crystals are usually found along the rim of the drop. They are very small, approximately one-twentieth the size of the larger square-shaped sodium chloride crystals. Sodium fluosilicate crystals are small, hexagonal, and under subdued light have a light pink hue. The test is sensitive to approximately 20 gammas of fluoride.

DETERMINATION OF DEATH BY DROWNING

The expert must often determine whether a person whose body is found in water met death by drowning or by some other cause. There are a number of tests described but none at present has any specific or reliable value. The surface signs of drowning have been suggested as means of proving this question.

1. Excessive coldness of corpse (of no value).
2. Extreme whiteness (of no value).
3. When the body is brought into contact with the air, it changes from brown to dark green more quickly on the breast than on the abdomen. The reverse occurs in all nondrowning cases (this sign is of no value).
4. Wrinkling of the skin (of no value because this may happen to a body thrown into the water after death).
5. Goose skin (this is not specific, because it is found in other conditions; it may also form after death).
6. Contraction of penis, scrotum, and breast (these may contract if the body is thrown into the water after death).
7. Foam in mouth and nose (this is found very often, although it is not a constant sign; it may, however, occur from other causes, as in pulmonary edema).
8. Ecchymoses in the conjunctivae and in the skin of the face (a rare finding).

The following interior signs have been suggested:

1. Dark and fluid blood (this is found in many other conditions).
2. Dilated thorax and distended abdomen (of no value).
3. Epiglottis relaxed (this is found in many other conditions).
4. Bladder empty (of no value).
5. Congestion of all organs (of no value; characteristic of all forms of asphyxia).
6. Excessive oozing of blood when skull cap is removed (of little value; characteristic of asphyxia in general).
7. Drowning fluid in stomach (Liman proved that water can get into the stomach after death, but in a small amount).
8. Large, pale red ecchymoses on surface of lungs (this is not a constant finding).
9. Drowning fluid in duodenum, jejunum, and even in the ileum (Kolisko). This finding is more specific than water in the stomach. There are numerous drowning cases, however, in which the water does not get into these parts of the alimentary tract. The presence of fluid in the ileum is typical of drowning because it cannot get there after death except by enormous pressure. However, it must not be overlooked that the absence of drowning fluid in the ileum does not mean that the case is not one of drowning, because in many cases the water does not reach the ileum.
10. The presence of fluid in the middle ear is significant only if the ear drum is intact; it has, however, been proved that this may happen after death.

11. Drowning fluid and foam in the trachea and lungs.[1] The fluid does not seem to get into the lungs until the third stage; then it goes to the bronchi and alveoli. The amount depends on the length of the terminal inspirations (there are exceptional cases in which there is no third stage). It is important in this connection to bear in mind the presence of foamy fluid in the lungs in cases of pulmonary edema.

12. Balloon-like lungs, increased in volume (this is not a constant finding).

13. Pieces of lungs sinking when thrown into water (of no value).

14. Inflation of lungs with colored liquids or gases to determine whether they will penetrate throughout (nondrowned lungs) or only partially (drowned lungs). This contention has been proved erroneous.

15. The presence of foreign bodies (present in the water) in the alveoli (Lesser). This is not a constant finding in drowning cases.

As Gettler[2] well states: "Most conditions named above are misleading and useless." The following three conditions will aid in deciding the cause of death; namely, (1) foamy fluid in the trachea, bronchioles, and lung cells; (2) microscopic finding of foreign bodies (from the water) in the bronchioles and alveoli; and (3) water in the stomach, duodenum, jejunum, and ileum.

Gettler and others turned their attention to chemical and physical methods for proving the question. They based their investigations on the well-known physiologic fact that blood from the lungs passes to the left heart and then through the organism, whereupon it goes to the right heart, and then back to the lungs. Water entering the lungs will quickly reach the left heart and dilute the blood therein, while the blood of the right heart remains relatively undiluted. Wachholz has proved experimentally, both in man and in animals, that no water can get into the left heart if the individual is thrown into the water after death. A number of methods have been suggested, but none has been so well proved as that reported by Gettler. He suggests the determination of the chloride content of the blood to determine the question at issue, since sea water contains a large amount of chloride and fresh water a negligible amount and that the chlorides of the inorganic constituents of the body are not likely to be affected by postmortem changes. Furthermore, the microchemical methods for chlorides lend themselves admirably to this work.

Chloride Test of Blood to Establish Drowning As Cause of Death

The method in detail advocated and used by Gettler very successfully as Pathologic Chemist for the Chief Medical Examiner of New York is as follows:

The pathologist should be supplied with two clean, dry glass bottles of about 30 c.c. capacity, labeled, respectively, right and left (no anticoagulant is used); two clean, dry 25 c.c. pipettes from which the tips have been broken off and the ragged edges rounded in a flame (the object of this is to have a large opening so that there is less chance of a small clot obstructing the opening); also a rubber tubing with glass mouthpiece for attaching to pipettes when drawing up the blood. The heart, while still in place in the cadaver, is wiped dry with a clean towel, and a small slit is made with a clean, dry knife in the right ventricle. The pipette with rubber attachment is then inserted into the heart, through the incision, and careful suction is applied. If the blood does not enter the pipette readily, the latter is carefully moved into different positions within the heart chamber until the end of the pipette rests within the blood. When from 5 to 10 c.c. of blood are in the pipette and while suction is still being applied, the rubber tubing attached to it is pinched tightly to prevent the blood from returning to the heart. The pipette is then drawn from the heart, and the contents are emptied into the vessel marked "right." This vessel should be stoppered, labeled, and sealed. With another dry knife, the left chamber of the heart is opened and the contents are put into the bottle marked "left," exactly the same technic being used.

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[1] The time interval between submersion and death in cases of drowning may be divided into three stages: First, the period in which respiration is held back. This lasts only a few seconds and is instinctive; it may be reflex and may result in prolonged respiratory suspension. Second, the stage of dyspnea, which consists in short but deep inspiration, quickly followed by expiration. This is followed by convulsive expiration. Third, the stage of asphyxiation. Here we have unconsciousness and lost reflexes and deep inhalation at long intervals.

[2] J. A. M. A. 77: 1650, 1921.

The Analysis in Detail.—

Pipette carefully 3 c.c. of the blood into a clean, dry Erlenmeyer flask; if the blood is badly clotted, accurately weigh 3 gm. Add 27 c.c. of distilled water and 30 c.c. of saturated picric acid. Mix well and let stand ten minutes; then filter through dry filter paper. Pipette accurately 40 c.c. of this filtrate into another dry Erlenmeyer flask and add 10 c.c. of M/29.25 silver nitrate solution. Mix thoroughly and allow to stand, preferably overnight. Then filter and pipette 20 c.c. of this filtrate into a clean casserole. Add 4 c.c. of the starch-citrate mixture and titrate with the standard M/117 potassium iodide solution. (Austin and Van Slyke.)

Calculation.—

$$125 \times \left(8 - \frac{\text{c.c. KI used}}{2}\right) = \text{mg. sodium chloride in 100 c.c.}$$

The **silver nitrate solution** contains, per liter, 5.812 gm. of $AgNO_3$ + 250 c.c. of concentrated nitric acid.

The **starch-citrate** solution is made as follows: Dissolve 2.5 gm. of soluble starch in 500 c.c. of warm water; then add 446 gm. of sodium citrate and 20 gm. of sodium nitrite and heat until all are dissolved. Filter while still hot through cotton; allow to cool and make up to 1 liter.

The **M/117 potassium iodide solution** contains 1.419 gm. in 1 liter.

Alternate Method

For precipitating the protein and formed elements, tungstic acid is used, and the technic of Folin and Wu is followed. This method is as accurate as the picric acid method:

Pipette 5 c.c. of blood into a dry Erlenmeyer flask; add 35 c.c. of water; mix thoroughly. Then add 5 c.c. of 10% sodium tungstate, mix well, and add 5 c.c. of $\frac{2}{3}$ normal sulphuric acid. Stopper with a well-fitting rubber stopper and shake vigorously for five minutes. Filter through dry paper into a dry beaker. Pipette 20 c.c. of the filtrate into another clean, dry beaker, add 10 c.c. of M/29.25 silver nitrate solution, mix well, and let stand, preferably overnight. Then filter through dry paper and pipette 15 c.c. of this filtrate into a casserole. Add 4 c.c. of the starch-citrate mixture, and titrate with M/117 potassium iodide solution.

Calculation.—

$$100 \left(10 - \frac{\text{c.c. KI used}}{2}\right) = \text{mg. sodium chloride in 100 c.c.}$$

The chloride content in the blood of the right and left chambers is the same in the various pathologic conditions so far examined, the greatest difference being 5 mg. in 100 c.c. of blood. In some cases the left is higher and in others the right; but the difference is never greater than 5 mg. In all those cases in which the drowning occurred in salt water, the chloride content is higher in the left heart chamber; the difference between the chloride content in the left and right heart chambers was marked by Gettler as plus. In those cases in which drowning occurred in fresh water, the left heart chamber always showed the lower chloride content and the difference is marked minus in his table.

Some cases have as high a difference as 294 mg., while others have only from 25 to 50 mg., depending on the amount of water going into the lungs and also on the time interval between the entrance of water into the lungs and death. The longer this time interval, the more water passes to the left heart chamber. Whenever the chloride content in the two chambers is so close as to give a difference of only 25 mg., it is advisable to repeat carefully the determination. If the same results are obtained a second time, one can safely assume that the case is one of drowning. It must be borne in mind, however, that it is possible in an exceptional case, for a person to die through shock as soon as he hits the water, or he may die in the first of the three drowning stages. If this is the case, then the chloride content is not altered.

DETECTION OF SILICON IN LUNGS

Of increasing importance is pneumonoconiosis, which is a disease due to breathing air containing silica (silicon dioxide). This disease, also called ''silicosis,'' shows anatomically generalized fibrotic changes and miliary nodulation in both lungs.

Crystalline and amorphous silicon compounds, comprising rock crystal, quartz, quartzite, flint, and sandstone, are the chief producers of silicosis. Lemon and Higgins[1] emphasize the point that more than 10 million particles of silica per cubic foot of air and a size of less than 10 microns are essential to any appreciable silicosis hazard. As high as 92 million particles have been determined. It has been shown that following inhalation the particles are engulfed by macrophages or ''dust cells'' which enter and block the lymph spaces. Mavrogordato believes that silica kills the dust cells and dissolves in the alkaline tissue juices forming a poisonous hydrosol jell ($Si(OH)_4$). The sharp edges of the silica particles are of very little importance in producing the changes in the lungs.

In the final analysis of proof that a given case is or is not silicosis at the autopsy table, resort must be made to a chemical analysis of the lungs for silica. According to McNally,[2] silica is a normal constituent of the human body but any lung containing more than 2 mg. of silicon dioxide per gm. has had undue exposure to a dusty atmosphere. In an investigation of a number of cases he found an average of 1.13 mg. of silica per gram of dry lung in non-silicotic persons. He found from 2.4 to 26.0 mg. of silicon dioxide per gram of dried lung in workers in various dusty trades. Stone and granite cutters showed the higher amounts. Church found the ash of potters' lungs to contain 47.78 per cent silica. Gardner[3] found almost universal microscopic but no gross evidence of silicosis in a study of 15 tunnel rock drillers and sand blasters and pulverizers, 12 of whom died of pulmonary infections within nine to eighteen months after short exposures. Sixty-one per cent of the bronchial lymph nodes were silicotic. The nodules varied from 0.3 to 0.4 mm. in diameter as compared with 3.0 to 4.0 mm. sizes found in hard rock miners and lead miners with many years' exposure. A maximum of 3.1 per cent silica content in the lungs was found.

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[1] Am. J. M. Sc. 719: 153, 1932.

[2] J. A. M. A. 101: 584, 1933.

[3] Am. J. Pub. Health 23: 1240, 1933.

Test for Silicon in Lungs, McNally Method[2]

Weigh and dry fresh material, pulverize, and determine a second moisture content on 20 gm. of the ground sample. Ash, weigh, and digest the sample with hydrochloric acid, evaporate to dryness, and repeat the process. Take up the residue with hydrochloric acid and water, filter, and incinerate the insoluble residue in a platinum dish.

This is recorded as the hydrochloric acid insoluble.

This ash is nearly always white. Residue is treated with hydrofluoric acid.

The loss of weight is recorded as silicon dioxide.

In the majority of instances, a faint to a weighable residue of iron-stained material is likely to be noticed. This is dissolved in hydrochloric acid and added to the original filtrate.

The percentage of moisture in the lung, the percentage of ash, and hydrochloric acid insoluble of ash are found in Table 233, below, from McNally's article.

TABLE 233.—PERCENTAGE OF MOISTURE IN THE LUNG, PERCENTAGE OF ASH, AND HYDROCHLORIC ACID INSOLUBLE OF ASH

Case	Moisture, Per Cent	Ash, Per Cent	Weight of Lung, Gm.	Mg. of SiO_2 per Gram of Dried Tissue
312	85.72	5.61	600.5	0.92
311	89.15	6.71	729.5	1.86
317	3.96*	4.51	----	1.22
325	86.28	5.61	306	1.06
324	89.55	7.19	785	0.90
323	77.15	4.66	652	0.85
333	88.04	6.23	518	1.10
332	83.34	5.35	893.2	3.28
39	88.42	5.67	402	2.21
41	73.96	3.75	619	1.65
35	85.31	---	388	1.40
42	84.21	6.58	602	1.50
34	89.13	5.52	561	4.60
350	89.98	5.14	707	0.92
352	86.20	7.51	708	2.40
349	82.57	3.82	1,048	0.73
267	86.08	8.13	468	7.06
359	81.99	5.24	543	0.79
356	87.18	5.39	397	1.41
357	89.37	5.77	416	1.25
360	89.87	5.98	304	2.66
358	81.82	3.66	122	0.13
368	85.84	3.36	312.5	1.51
361	84.49	5.97	321	2.37
337	81.79	5.68	756	9.75
389	89.13	6.51	310	0.64
431	90.06	5.15	780	0.71
441	86.03	6.17	378	1.21
455	----	4.76	---	1.80
459	----	4.10	---	0.40

*Moisture on dried sample.

The following example is of a specimen received in our laboratories.

Weight of crucible plus wet sample of lung	43.593 gm.
Weight of crucible empty	17.073 gm.
Weight of wet sample	26.520 gm.

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[2] J. A. M. A. **101**: 584, 1933.

Sample was then dried.

Weight of dried sample plus crucible	23.593 gm.
Weight of crucible empty	17.073 gm.
Weight of dried sample	6.520 gm.

Sample was then pulverized, dried and reweighed.

Weight of pulverized sample plus crucible	23.526 gm.
Weight of crucible empty	17.073 gm.
Weight of pulverized dried sample	6.453 gm.

Sample was then ashed.

Weight of crucible plus ash	17.379 gm.
Weight of crucible empty	17.073 gm.
Weight of ash	0.306 gm.

Ash treated with hydrochloric acid, filtered, and ashed on filter paper.

Weight of insoluble residue plus paper ash plus crucible	17.156 gm.
Weight of paper ash	0.002 gm.
Weight of residue plus crucible	17.154 gm.

Weight of crucible plus residue	17.154 gm.
Weight of crucible empty	17.073 gm.
Weight of hydrochloric insoluble	0.081 gm.

Residue treated with hydrofluoric acid, diluted with water, filtered, and ashed.

Weight of residue plus paper ash plus crucible	17.078 gm.
Weight of paper ash	0.002 gm.
Weight of residue plus crucible	17.076 gm.

Weight of crucible plus residue	17.076 gm.
Weight of crucible empty	17.073 gm.
Weight of residue	0.003 gm.

Weight of insoluble hydrochloric acid residue	0.081 gm.
Weight of hydrofluoric acid residue	0.003 gm.
Weight of SIO_2	0.078 gm.

Calculations and Results.—

26.520	gm.—weight of lung
6.453	gm.—weight of dried lung
20.067	gm.—loss of water.

$\frac{20.067}{26.520} = 75.66\%$ of water.

Weight of dried tissue	6.453 gm.
Weight of ash	0.326 gm.

$\frac{0.326}{6.453} = 5.05\%$ ash in dried lung tissue.

$\frac{0.078}{6.453} = 0.0121$ gm. of silicon dioxide per gram of dried lung tissue

or

12.1 mg. of silicon dioxide per gram of dried lung tissue.

Spectrographic Test for Silicon

Once more may we suggest the superiority of spectrographic determination of silicon over previously described chemical methods. They are simple and exact.

DICHLORO-DIPHENYL-TRICHLORETHANE (DDT)

Smith and Stohlman[1] reported a method of testing for dichloro-diphenyl-trichloroethane (DDT) which may be applied in cases of possible poisoning with DDT. The method is applicable to the original powder and to blood, urine, feces, and tissue.

Extract a definite weight of DDT powder with several portions of acetone.

Collect the extracts and evaporate to dryness on a water bath.

Dissolve the residue in 10 c.c. of absolute alcohol and transfer quantitatively to a reflux condenser.

Add 1 gram of metallic sodium, cut into small bits.

Reflux for one-half hour.

Transfer the contents of the flask quantitatively with the aid of distilled water to an Erlenmeyer flask.

Acidify with 3 c.c. of concentrated nitric acid.

Decolorize with 1 gram of chlorine-free Nuchar (charcoal).

Filter.

Titrate the filtrate for chlorides using either the Volhard-Arnold or the Schales method (pages 433 and 435).

Calculations.—

Calculate the chlorine (*not* the sodium chloride). Multiply the result by 2 to convert to DDT, since 50% of the weight of DDT is Cl.

Example.—

The specimen contained 6 mg. of chlorine by this test.

2 × 6 = 12 mg. of DDT present.

DDT in Body Tissues or Fluids

Extract body tissues or fluids by means of steam distillation (page 2243).

Treat the residual mash for purification in the same manner as for the barbiturate group (page 2285), substituting for the acid-ether extraction an extraction with several portions of acid-petroleum ether (benzine).

Evaporate the combined petroleum ether extracts.

Pick up the residue with acetone.

Proceed as for testing for DDT above.

The general opinion regarding toxicity of DDT is that from 150 to 200 mg. of DDT per kilogram body weight is toxic for man. The few cases of fatal poisoning due to DDT which have been reported in the literature were all due to accidental ingestion or inhalation of the substance. These accidents occurred during the process of applying DDT in an effort to exterminate insects.

BENZEDRINE

Since benzedrine does not undergo deamination in the human body, a direct determination may be made in body fluids. Benzedrine is chemically akin to ephedrine and adrenaline.

In Urine and Viscera

Acidify the urine or an extract of the viscera with tartaric acid.

Carry out steam distillation as described on page 2243.

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[1] Pub. Health Rep. 59: 984, 1944.

Collect the distillate into 10 c.c. of N/1 hydrochloric acid.

Distill for approximately 1 hour.

Add sodium hydroxide solution to the benzedrine-hydrochloric acid mixture until it is alkaline.

Extract with several small portions of ether.

Collect the extracts, and add to the combined extracts a few drops of concentrated hydrochloric acid and 5 c.c. of alcohol.

Evaporate to dryness on a steam bath. Carry out the tests below.

In Benzedrine Tablets

Crush the tablet. Mix with it a little solid barium chloride and 1 c.c. of N/1 hydrochloric acid.

Add 25 c.c. of absolute alcohol.

Heat on a water bath for several minutes.

Filter.

Wash the residue with absolute alcohol, and save the alcohol extracts.

To the combined alcohol extracts add 1 c.c. of N/1 hydrochloric acid.

Evaporate with gentle heat to almost dryness on a steam bath.

Dissolve the residue with a small quantity of N/1 hydrochloric acid.

Filter and transfer quantitatively into a separatory funnel.

Make the filtrate slightly alkaline with 10% sodium hydroxide solution.

Extract with chloroform.

Separate off the chloroform layer and add a few drops of N/1 hydrochloric acid, then evaporate the chloroform extracts to dryness. Carry out the tests below.

Tests for Benzedrine

Benzedrine and Pervitin (n-methyl benzedrine) give the general reactions for alkaloids.

Dissolve the residue (above) in 20 drops of water. Place 2 drops of the solution on each of several slides. Carry out tests on known benzedrine samples simultaneously.

(A) Add 1 drop of 5 per cent chloroplatinic acid solution. Typical pale yellow crystals will form if benzedrine is present. Examine under the microscope. Compare with control crystals.

(B) Add 1 drop of Marquis reagent to the drops on the slide. If benzedrine is present a brick red precipitate will form, which persists approximately only 30 seconds. A dirty brown colored precipitate forms and persists approximately 1 hour. A dirty olive green precipitate forms.

(C) Benzedrine gives the usual precipitation reactions with Mayer and Dragendorff reagents below.

Marquis Reagent.—

Several drops of formaldehyde in 5 c.c. of concentrated sulphuric acid.

Mayer Reagent for Alkaloids.—

Dissolve 13.55 gm. mercuric chloride
and 50 gm. potassium iodide in distilled water and dilute to 1000 c.c. with distilled water.

A white precipitate forms with most alkaloids in acid solution.

Dragendorff Reagent for Alkaloids.—

Dissolve 8 gm. of bismuth subnitrate in 20 c.c. of nitric acid.

Add the solution gradually to a concentrated solution of 22.7 grams of potassium iodide in a small amount of water.

Cool. Decant the fluid from the potassium nitrate crystals and dilute to 100 c.c. with distilled water.

Most alkalies give a reddish flocculent precipitate with this reagent.

TABLE 234.—PROPERTIES OF SOME IMPORTANT SOLVENTS USED IN INDUSTRY**

SOLVENT	COMMERCIAL SYNONYM	LIMITS OF INFL. BY VOL. %		EXPLOSIVE RANGE	SPECIFIC GRAVITY		B. P. ° C.	TOXIC LIMIT*	HAZARDS			PHYSIOL. ACTION
		LOWER	UPPER		SP. GR.	COMP. WITH AIR			FIRE	EXPLOSION	DERMATITIS	
Acetone	Dimethyl ketone	2.5	12.8	10.3	.790	2.0	56	200 P.P.M. (1)	+	+	+	Irritant, dizziness
Amyl acetate	Banana oil	1.1		(F.P.-94° F.)	.868	4.5	147.6	400 P.P.M. (2)	+		+	Intox., rapid pulse
Aniline	Aniline oil			(F.P.-131° F.)	1.022	3.22	184.4	5 P.P.M. (3)				Narcotic, upset stomach
Benzene	Benzol	1.4	6.8	5.4	.879	2.7	80	100 P.P.M. (4)	+	+	+	Anemia, headache, dizziness
Benzine	Petroleum vapors	1.1		(F.P.-0° F.)	.625	4.48	35-80	1000 P.P.M. (12)	+	+	+	Headache, vertigo, nausea
Butanol	Butyl alcohol	1.7		(F.P.-115° F.)	.811	2.55	117.7	100 P.P.M. (5)	+		+	Liver degeneration
Butyl acetate	n. butyl acetate	1.7		(F.P.-100° F.)	.883	4.0	126.5	400 P.P.M. (6)	+		+	Irritates eyes, nose, throat
Carbon tetrachloride	Tetrachlormethane	(Noninflam.)			1.60	5.3	76.8	100 P.P.M. (7)			+	Irritates nose, eyes, throat
Chlorobenzene	Monochlorobenzene			(F.P.-81° F.)	1.106	3.8	132	75 P.P.M. (14)	+	+	+	Narcotic
Ethanol	Alcohol	2.3	19.0	15.7	.789	1.6	78	250 P.P.M. (8)	+		+	Resp. irritant, anesthetic
Ethyl ether	Ether	2.3	6.15	3.85	.708	2.55	34.4	400 P.P.M. (9)	+	+	+	Anesthetic
Ethylene dichloride	Dutch liquid	6.2	15.9	9.7	1.25	3.42	83.5	100 P.P.M. (10)			+	Dizziness, nausea, narcosis
Gasoline		1.5	6.0	4.5	.747	3-4	50-140	1000 P.P.M. (19)	+	+	+	Headache, vertigo, nausea
Methanol	Wood alcohol	6.7	36.5	29.8	.793	1.1	64	100 P.P.M. (20)	+		+	Nerve poison, irritant
Naphtha	Stoddard solvent	1.2	6.0	4.8	.788	4.9	156	5000 P.P.M. (21)	+	+	+	Headache, vertigo, nausea
Perchloroethylene	Tetrachlorethylene	(Noninflam.)			1.608	5.72	122	100 P.P.M. (11)			+	Irritation to eyes, dyspnea
Tetrachlorethane	Westron	(Noninflam.)			1.60		130.5	10 P.P.M. (13)			+	Narcotic, liver damage
Tetrachloroethane	Westrosol	(Noninflam.)			1.46	4.5	86.7	100 P.P.M. (16)			+	Nervous disturbances
Toluene	Toluol	1.3	6.8	5.5	.866	3.2	111	100 P.P.M. (15)	+			Renal irritation
Turpentine	Spirits of turpentine	0.8		(F.P.-95° F.)	.860		116.7	200 P.P.M. (17)	+		+	Irritates eyes, nose, upper resp.
Xylene	Dimethylbenzene	1.0	6.0	5.0	.88	3.68	139	100 P.P.M. (18)	+	+		Asphyxia

*NOTE: Recommended by following states: (1) Cal., Colo.; (2) Cal., Colo., Kan., Ky., Mass., Minn.; (3) Cal., Colo., Kan., Mass., Minn., Okla.; (4) Cal., Colo., Conn., Penna., S. C.; (5) Cal., Colo., Kan., Okla.; (6) Cal., Colo., Kan., Ky., Mass., Minn., Okla., Penna., Wis.; (7) Cal., Colo., Conn., Kan., Ky., Md., Mass., Minn., Okla., Penna., S. C., Wis.; (8) Cal., Colo.; (9) Cal., Colo., Kan., Mass., Mich., Minn., Okla., Wis.; (10) Cal., Colo., Kan., Mass., Mich., Minn., Okla., Wis.; (11) Mich.; (12) Mich.; (13) Cal., Colo., Kan., Ky., Mass., Minn., Okla., Wis.; (14) Cal., Colo., Kan., Mass., Minn., Okla., Wis.; (15) Cal., Colo.; (16) Mich.; (17) Kan., Mass., Mich., Minn., Okla., Wis.; (19) Cal., Colo., Conn., Kan., Ky., Md., Mass., Minn., Okla., S. C., Wis.; (20) Cal., Colo., Conn., Ky., Md., S. C.; (21) Cal., Colo. (Compiled by A. E. Dooley, Penna., Health Dept.)

**Compiled by A. Rothman, U. S. Army.

Morgan[1] stated that benzedrine can be detected in urine for three to four days after a fairly large injection of the substance. It is eliminated slowly in the urine; thus the unit concentration may be low. Approximately 50 per cent is eliminated in the first 48 hours. Highest recoveries may be obtained within the first one to eight hours after injection.

Properties of Some Important Solvents Used in Industry

Refer to Table 234.

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[1] Personal communication.

CHAPTER XVI

BASAL METABOLISM

The terms "Basal Metabolism" and "Basal Metabolic Rate" mean the heat production of an organism measured from twelve to eighteen hours after the ingestion of food with the organism at complete muscular rest. This may be determined directly by actual measurement by means of the calorimeter or indirectly by calculating heat production from an analysis of the end products which result from oxidation within the organism, or from the amount of oxygen used.

There are certain well-known facts about metabolism which must be considered. We know that food is taken into the gastrointestinal canal, there prepared for absorption, thence carried to the tissues either to be directly consumed or else to be stored away. Waste products are formed and excreted from the body. By comparison of the products excreted with those taken in as food, we know how much is retained or lost. This constitutes general metabolism. Special metabolism entails the study of the chemical changes which each of the foodstuffs undergoes in passing through the phases of absorption and excretion.

The methods of determination of basal metabolism are: first, gas analysis; second, the estimation of oxygen used and carbon dioxide eliminated; and third, the estimation of the amount of oxygen used, at a given time, or the length of time necessary to use a given amount of oxygen.

General Instructions for Basal Metabolism Patients

The patient is instructed to eat a light dinner, to have a good night's rest, and to report to the laboratory in the morning without breakfast. If the patient is in the hospital, either the apparatus is taken to the bedside, or the patient is wheeled into the basal metabolism room. Undue exercise is avoided.

If the patient reports to the laboratory for the test, he is allowed to rest for thirty minutes to one hour. During this time, he is not to be disturbed by conversation or loud noises. The room in which the patient is resting should be away from those parts of the building in which any disturbances or noises may occur.

The basal metabolism apparatus is prepared away from the patient and taken to the side of the bed after preparation. Do not set up the machine before the patient.

The patient should be told something about what is expected of him before beginning the test. It is necessary only to explain to him that he is to breathe naturally into the apparatus, and that the technician is simply going to measure the quantity of oxygen that he is consuming. Frequently it is necessary to make one or two preliminary tests to acquaint the patient with the apparatus.

The mouthpieces and the nose clamp should be washed with hot water and soap, thoroughly rinsed, and disinfected before beginning the test. Place them in alcohol for fifteen minutes before using. Rinse many times with tap water before using on the patient.

The soda lime (4-8 ''Wilson'' soda lime, if the Collins machine is used, or Baralyme for the Sanborn Metabulator) in the apparatus should be changed from time to time, and should never be used up to the point of becoming wet. About 50 tests can be made without changing the lime.

An extra tank of oxygen should be kept on hand. Be sure to read both the outside and inside labels of the tank to make certain that you are using an oxygen tank. Other gases, such as nitrous oxide, have been sent to laboratories by mistake with serious consequences.

Basal Metabolism

Basal metabolism determinations are very important in a wide range of diseases. The basal metabolic rate is, of course, most important in diseases of the thyroid gland. Extremely high rates are seen in cases of toxic goiter, both in adenomata with hyperthyroidism and exophthalmic goiter. The more severe the thyrotoxicosis, the greater the rate will be. It is generally accepted that a basal metabolic rate of over +40 is sufficient grounds for postponing operation until this rate has been materially reduced.

The highest rates are seen in cases of exophthalmic goiter. In certain cases of thyrotoxicosis of a borderline type, it is necessary to conduct a therapeutic test on iodides in order to establish the diagnosis. We make two or three determinations of basal metabolic rate before any iodide is administered. If any degree of thyrotoxicosis is present, subsequent tests done after five to ten days with patient on iodides will show lowering of basal metabolic rate of 15% or more.

In cases of nontoxic goiter, it is necessary to control these cases by basal metabolic rate determinations so as to know when the transition takes place from nontoxic to toxic goiter. In cases of nontoxic goiter due to adenomata without any symptoms of hyperthyroidism, if the basal metabolic rate is 10 to 20 per cent above normal, hyperthyroidism should be suspected. This enables us to control the case long before the heart and the nervous system become damaged. In cases of nontoxic colloid goiter, the basal metabolic rate is usually within normal limits. In myxedema, where there is a partial or complete atrophy of the thyroid gland, the basal metabolic rate is between –35 and –45. In cases of cretinism, the basal metabolic rate is low.

The basal metabolic rate is useful in differentiating cardiorenal disease from myxedema with which it is often confused. In myxedema the rate is always below –20, whereas nephritis does not cause these low rates.

Dyspnea causes an increase of the metabolic rate; when edema exists with cardiorenal disease, the basal metabolic rate falls after the initial rise caused by the dyspnea itself.

There are a number of cases in which the basal metabolic rate serves to establish the case as a thyrotoxic one rather than primarily a cardiac case.

Toxic goiter causes many forms of irregularities of the heartbeat, especially auricular fibrillation. In these cases the basal metabolic rate will furnish the necessary evidence by showing a plus rate. In the so-called "effort syndrome" the basal metabolic rate is usually normal. However, a primary test on such a case may show a rate of +10 to +25 because of the extreme nervousness of the patient, but subsequent tests will show the rate to approach normal limits.

Dysfunction of all the endocrine glands affects the basal metabolic rate. In anterior lobe pituitary disease, resulting in acromegaly or gigantism, the basal metabolic rate is slightly increased in the early stages of the disease. In disease of the posterior lobe, the basal metabolic rate is either normal or slightly increased. In lesions of the pituitary alone, or in combination with lesions in the sella turcica, causing Fröhlich's syndrome, the basal metabolic rate is usually decreased.

In atrophy of the adrenal glands, seen in Addison's disease, the basal metabolic rate is low.

In gonadic disturbances where there is loss of the ovarian hormone, due to ovariectomy or to the onset of menopause, there is a low basal metabolic rate.

In certain types of leukemia of myelogenous or splenic origin, the basal metabolic rate is increased. Polycythemia shows an elevated basal metabolic rate. Pregnancy shows a basal metabolic rate as high as +30% after the fifth month.

In certain types of obesity, resulting from disturbance of the endocrine glands, the basal metabolic rate is low. The test serves to distinguish between incipient tuberculosis and certain diseases resembling this condition. In diabetes of a severe type the rate is lower than normal.

In general, it may be stated that fever increases the basal metabolic rate and that if a patient has fever, the normal for that patient should be increased about 7.2 per cent for each degree of fever.

THE COLLINS (BENEDICT-ROTH) APPARATUS

Description of the Apparatus

The apparatus is placed on a table or a stand by the bedside. Adjustment of the mouthpiece to the proper height is obtained by raising or lowering the horizontal support. The metal mouthpiece assembly slides on the horizontal bar, and can be easily removed for sterilization.

The thermometer may be removed when carrying the apparatus. It should be kept in place in the oxygen bell during the test, and the temperature recorded before and after each test.

A high-grade clockworks revolves a drum on which is placed the graph used in recording the test. On the upright arm of the apparatus in front of this drum is a pen. The pen is detachable. After using the pen, it is first washed thoroughly in water, then dried in alcohol and ether. The pen should be kept clean.

There are two rubber breathing tubes below the apparatus. The shorter piece of tubing is connected to the upper metal, and the longer piece to the lower metal connection below the apparatus.

There are two petcocks below the apparatus; one is for oxygen inlet, the other for the removal of the water from the apparatus after use. Each petcock is labeled.

To Use the Apparatus

Remove the oxygen bell. Fill the soda lime container with 4-8 "Wilson" soda lime and screw the container in place. Place the metal top on the can and seal with the wide rubber band supplied.

Fill the outside container with water up to within two inches of the top. This provides a seal against the escape of oxygen from the apparatus.

Fill the apparatus with oxygen.

Place the pen in position and fill with kymograph ink.

Place the kymograph paper around the drum. The bottom of the paper should be about ¼ inch above the bottom of the drum. Before placing the drum in position, be sure that the clock has been wound.

The vertical lines on the kymograph paper are placed at one minute intervals; the horizontal lines indicate mm. for detecting rise in O_2 line.

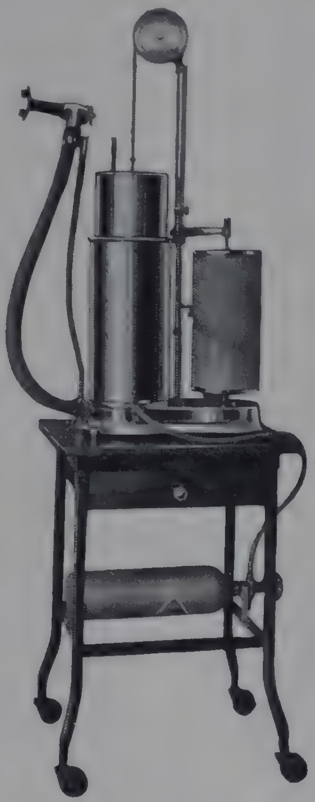


Fig. 719.—Collins (Benedict-Roth) basal metabolism apparatus. (Courtesy Warren E. Collins, Inc., Boston.)

The apparatus should be tested for leakage before attaching to the patient. This is accomplished by placing a few inches of oxygen in the apparatus and closing the mouthpiece with a rubber stopper. Next place a 200-gram weight on the bell. The pointer against the millimeter scale must remain stationary for a minute or more if there is no leak. Remove the oxygen and refill the oxygen bell with fresh oxygen for the test.

Test for obstructions in the breathing apparatus by breathing through the mouthpiece. If there is an obstruction, drain the absorber in the lower breathing tube.

Place the rubber mouthpiece on the apparatus, and instruct the patient how to put it in his mouth. Be sure that no oxygen or carbon dioxide can escape from the mouthpiece.

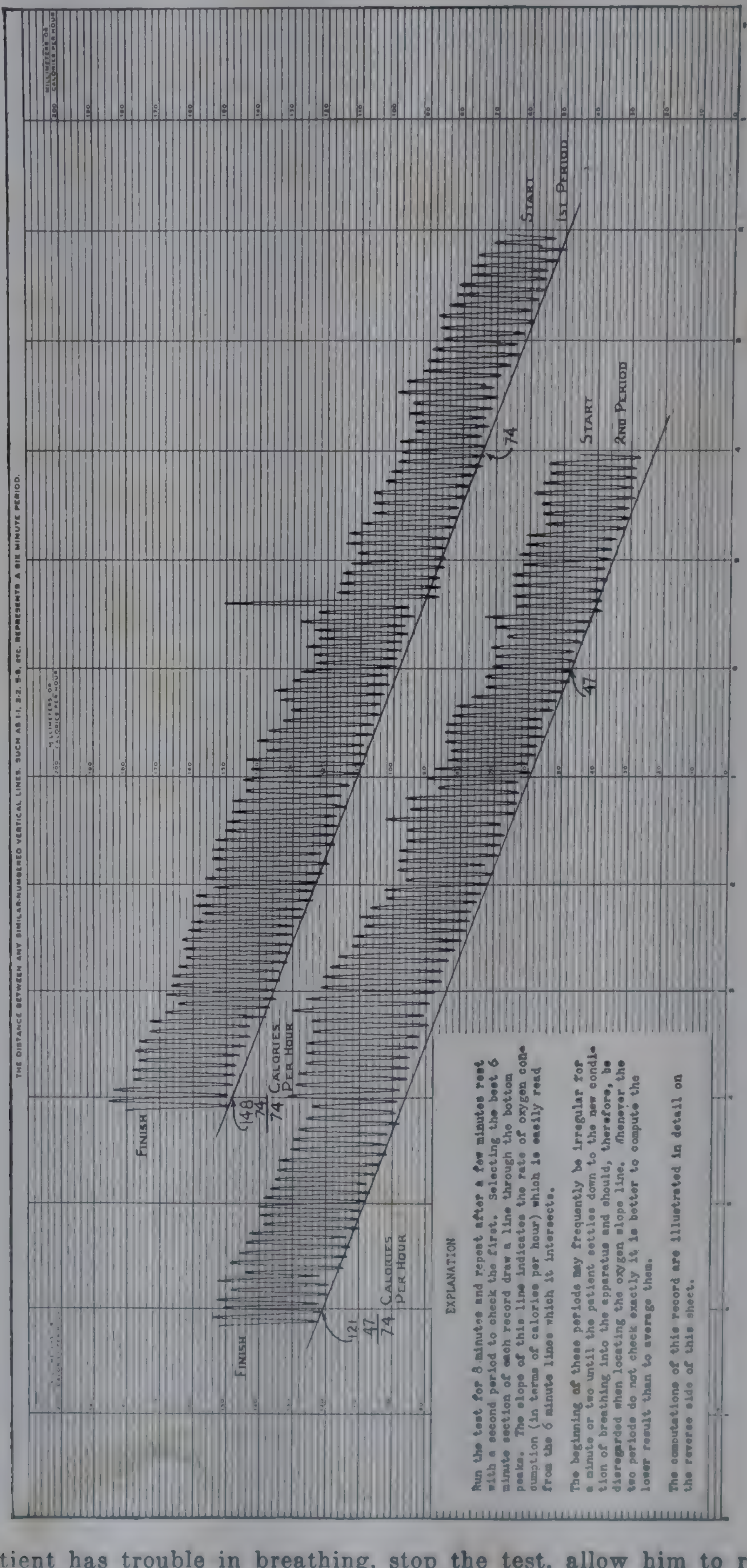
Apply the noseclip, and ask the patient to make a slight effort to breathe through his nose. If he can breathe through his nose, the clamp must be replaced in correct position.

Record the reading of the thermometer in the apparatus.

Swing the kymograph attachment around until the pen touches the paper, and allow the patient to breathe into the apparatus for about eight or ten minutes. The test requires six minutes' breathing, but it is sometimes necessary to make longer tests to accustom the patient to the apparatus.

If there is a leak of oxygen from the oxygen bell or any of the connections, the graph on the kymograph chart will rise very rapidly.

If the patient is breathing outside air, and not the oxygen in the tank, the line made by the pen will be almost horizontal.



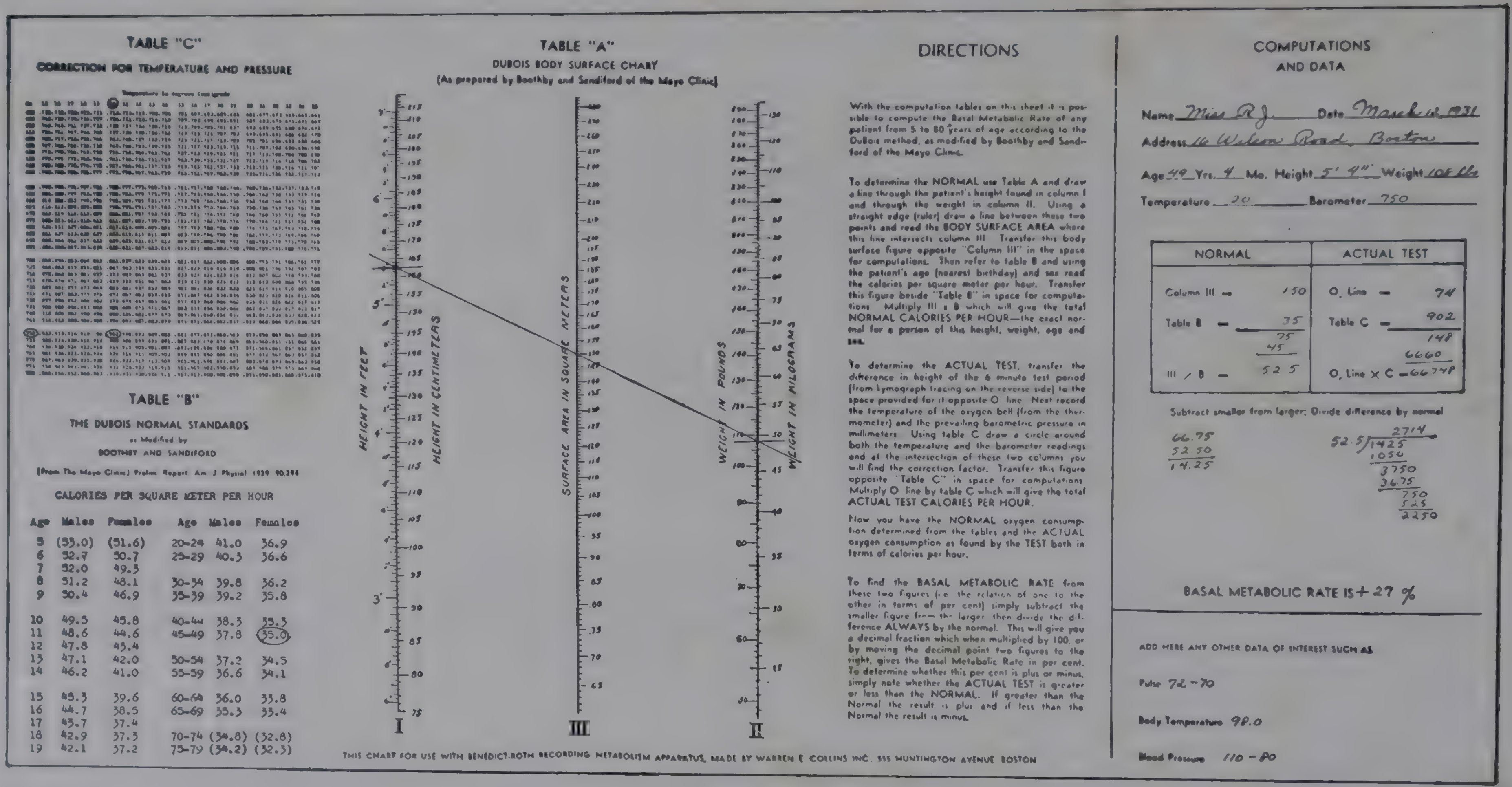
Run the test for 8 minutes and repeat after a few minutes rest with a second period to check the first. Selecting the best 6 minute section of each record draw a line through the bottom peaks. The slope of this line indicates the rate of oxygen consumption (in terms of calories per hour) which is easily read from the 6 minute lines which it intersects.

The beginning of these periods may frequently be irregular for a minute or two until the patient settles down to the new condition of breathing into the apparatus and should, therefore, be disregarded when locating the oxygen slope line. Whenever the two periods do not check exactly it is better to compute the lower result than to average them.

The computations of this record are illustrated in detail on the reverse side of this sheet.

Fig. 720.—(Courtesy Warren E. Collins, Inc., Boston.)

If the patient has trouble in breathing, stop the test, allow him to rest, and renew the effort to obtain regular breathing after a few minutes. Occasionally it may be necessary to make many tests before obtaining regular breathing.



HEIGHT IN FEET
HEIGHT IN CENTIMETERS
SURFACE AREA IN SQUARE METERS
WEIGHT IN POUNDS
WEIGHT IN KILOGRAMS
I
III
II

TABLE "C"

CORRECTION FOR TEMPERATURE AND PRESSURE

Temperature in degrees Centigrade

TABLE "B"

THE DUBOIS NORMAL STANDARDS

as Modified by

BOOTHBY AND SANDIFORD

(From The Mayo Clinic) Prelim. Report Am. J. Physiol 1929 90.298

CALORIES PER SQUARE METER PER HOUR

Age	Males	Females	Age	Males	Females
5	(53.0)	(51.6)	20–24	41.0	36.9
6	52.7	50.7	25–29	40.3	36.6
7	52.0	49.3			
8	51.2	48.1	30–34	39.8	36.2
9	50.4	46.9	35–39	39.2	35.8
10	49.5	45.8	40–44	38.3	35.3
11	48.6	44.6	45–49	37.8	35.0
12	47.8	43.4			
13	47.1	42.0	50–54	37.2	34.5
14	46.2	41.0	55–59	36.6	34.1
15	45.3	39.6	60–64	36.0	33.8
16	44.7	38.5	65–69	35.3	33.4
17	43.7	37.4			
18	42.9	37.3	70–74	(34.8)	(32.8)
19	42.1	37.2	75–79	(34.2)	(32.3)

TABLE "A"

DUBOIS BODY SURFACE CHART

(As prepared by Boothby and Sandiford of the Mayo Clinic)

DIRECTIONS

With the computation tables on this sheet it is possible to compute the Basal Metabolic Rate of any patient from 5 to 80 years of age according to the DuBois method, as modified by Boothby and Sandiford of the Mayo Clinic.

To determine the NORMAL use Table A and draw a line through the patient's height found in column I and through the weight in column II. Using a straight edge (ruler) draw a line between these two points and read the BODY SURFACE AREA where this line intersects column III. Transfer this body surface figure opposite "Column III" in the space for computations. Then refer to table B and using the patient's age (nearest birthday) and sex read the calories per square meter per hour. Transfer this figure beside "Table B" in space for computations. Multiply III x B which will give the total NORMAL CALORIES PER HOUR—the exact normal for a person of this height, weight, age and sex.

To determine the ACTUAL TEST, transfer the difference in height of the 6 minute test period (from kymograph tracing on the reverse side) to the space provided for it opposite O line. Next record the temperature of the oxygen bell (from the thermometer) and the prevailing barometric pressure in millimeters. Using table C draw a circle around both the temperature and the barometer readings and at the intersection of these two columns you will find the correction factor. Transfer this figure opposite "Table C" in space for computations. Multiply O line by table C which will give the total ACTUAL TEST CALORIES PER HOUR.

Now you have the NORMAL oxygen consumption determined from the tables and the ACTUAL oxygen consumption as found by the TEST both in terms of calories per hour.

To find the BASAL METABOLIC RATE from these two figures (i.e. the relation of one to the other in terms of per cent) simply subtract the smaller figure from the larger then divide the difference ALWAYS by the normal. This will give you a decimal fraction which when multiplied by 100, or by moving the decimal point two figures to the right, gives the Basal Metabolic Rate in per cent. To determine whether this per cent is plus or minus, simply note whether the ACTUAL TEST is greater or less than the NORMAL. If greater than the Normal the result is plus and if less than the Normal the result is minus.

COMPUTATIONS

AND DATA

Name Miss R. J. Date March 12, 1931

Address 16 Wilson Road, Boston

Age 49 Yrs. 4 Mo. Height 5' 4" Weight 108 lbs

Temperature 20 Barometer 750

NORMAL		ACTUAL TEST	
Column III —	150	O. Line —	74
Table B —	35 75 45	Table C —	902 148 6660
III / B —	52 5	O. Line x C —	66748

Subtract smaller from larger; Divide difference by normal

66.75
52.50
14.25

52.5)1425 2714
1050
3750
3675
750
525
2250

BASAL METABOLIC RATE IS + 27 %

ADD HERE ANY OTHER DATA OF INTEREST SUCH AS

Pulse 72 – 70

Body Temperature 98.0

Blood Pressure 110 – 80

THIS CHART FOR USE WITH BENEDICT-ROTH RECORDING METABOLISM APPARATUS, MADE BY WARREN E. COLLINS INC. 555 HUNTINGTON AVENUE BOSTON

Fig. 721.

NOTE: Table "C" is given here only to illustrate the method of obtaining the correction for temperature and pressure. This table will be found on the reverse side of all Collins kymograph sheets. (Courtesy Warren E. Collins, Inc., Boston.)

When the test is completed, again read the thermometer.

Obtain the patient's age, sex, weight without clothing, height without shoes.

Read the barometric pressure. All these facts are necessary for calculating basal metabolic rates.

At least two tests must be made on each patient, and the average used.

Calculation

Connect the majority of the **bottoms** of the kymograph lines by ruling a line between the **lowest** of these lines, and the bottom of the last kymograph line six minutes removed. If more than a six-minute test has been made, select the six minutes in the test which give evidence of the most regular breathing. The line is always drawn for a six-minute test.

The rise in millimeters of the O_2 line is the difference between the end of the line after six minutes and the beginning. For example, if one end of the line cuts the vertical line at 121 mm., and the other end at 47 mm., the rise in the O_2 line is $121 - 47 = 74$.

Figs. 720 and 721 give methods of computing basal metabolic rates of all patients from five to eighty years of age according to the method of Du Bois modified by Boothby and Sandiford of the Mayo Clinic.

To determine the **Normal***: Use Table A (Fig. 721). Rule a line to connect the ''patient's height'' found in Column I with the ''patient's weight'' found in Column II. The point at which this line intersects Column III is the *body surface area.* Record this in the computation space allotted to it. Now, using Table B, find the calories per square meter per hour by using the patient's age (nearest birthday) and sex, and record in space for computations. Multiply III by B to obtain total *normal calories per hour,* the exact normal for a person of this height, weight, age, and sex.

To determine the **Actual Test:** Record difference in height of the 6 minute test period from the kymograph tracing in the space called ''O_2 line.'' Record the temperature of the oxygen bell from its thermometer, and the prevailing barometric pressure in millimeters. Obtain correction factor for pressure and temperature from Table C (the intersection of the two columns representing the temperature and pressure for this test). Record in proper computation space. Multiply O_2 line by Table C to obtain *total actual test calories per hour.*

To find the **Basal Metabolic Rate:** Use the *Normal* and the *Actual* consumption of oxygen per hour. Subtract the smaller from the larger figure. Divide the difference by the *Normal.* Move the decimal two places to the right to obtain *Basal Metabolic Rate* in per cent. If the *Actual Test* is greater than the *Normal,* report as plus; if less than the *Normal,* report as minus.

SANBORN METABULATOR[1]

The Sanborn Metabulator is the latest equipment (1947) made by the Sanborn Company for making basal metabolism tests. (See Figs. 722 and 723.) Before attempting to cope with an actual patient it is a very sound policy to run trial tests on normal subjects.

Preparation of the Patient.—The patient is prepared in the usual manner. He must be completely at rest mentally and physically, and 14 hours must have elapsed since his last meal. He should lie down for one-half hour immediately preceding the test made with this equipment. (This is true of all equipment.)

Preparation of the Apparatus.—With the patient comfortable and resting, wheel the metabolism tester to a convenient position beside the bed and plug its power cord into the AC outlet. Place the chromium-plated leak tester in a glass of cold water and allow it to remain there while the patient is being prepared. Place a sterilized rubber mouthpiece on the mouthpiece connector. Open up the plexiglass window over the writing stylus. Turn the breathing valve to the ''test'' position, thus connecting the bellows with the outside air, and then collapse the bellows by pushing the bellows carriage toward the absorption chamber as far as it will go. With the bellows in this position, turn the patient valve back to ''fill,'' thus closing off the bellows. Now admit oxygen by carefully turning the handwheel on the oxygen tank to the left. Continue filling until the writing stylus is about an inch from the top edge of the paper; then close the valve on the oxygen tank tightly by turning it to the right.

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*These calculations are compiled from data furnished by Warren E. Collins, Inc., Boston, Mass.

[1] These data are compiled from facts furnished by the Sanborn Co., Cambridge, Mass. For further information, refer to the brochure supplied with each Metabulator.

Adjusting the Noseclip.—The instrument is now ready for the test. Connect the patient to the apparatus. Adjust the noseclip before inserting the mouthpiece. The sponge rubber pads are used to apply pressure against the soft sides of the nostrils. Loosen the thumbscrew so that the clip will fit loosely when first applied, and then take the noseclip between thumb and finger of both hands and, placing it over the nose near the bridge, slide it downward parallel to the patient's face until the pads are at the right level. Now tighten the thumbscrew until the patient finds he can no longer breathe out through his nose. Make sure of this by asking him to close his mouth and try. The noseclip must not be uncomfortably tight, for the patient will have to wear it for nearly ten minutes, but on the other hand, it must be tight enough to prevent any possible leakage. When the adjustment is satisfactory, remove the noseclip again by springing its arm outward slightly without changing the adjustment of the thumbscrew.

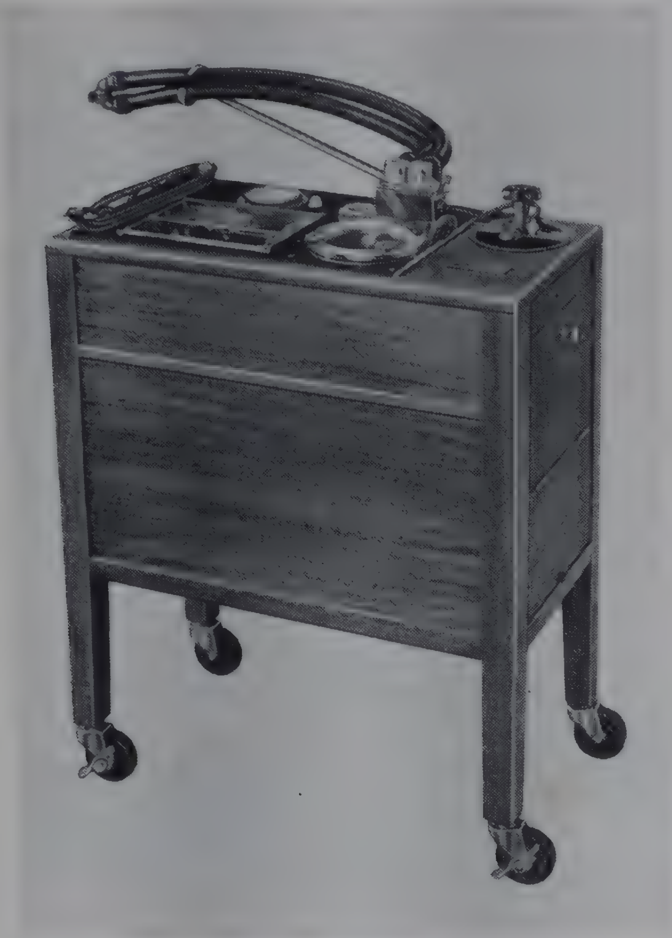


Fig. 722.—Sanborn basal metabolism apparatus. (Courtesy Sanborn Company, Cambridge, Mass.)

Placing the Mouthpiece.—Adjust the portion of the tester and of its flexible arm until the rubber mouthpiece is just in front of the patient's mouth, with its long axis pointing across the face. Insert the mouthpiece so that the two small projections lie between the upper and lower teeth while the flange of the mouthpiece lies between the lips and the teeth. See that the lips are not folded under, for this may permit leakage.

False Teeth.—False teeth will not cause trouble, especially if the teeth fit well. Since there may be an air passage over the plate, however, it is doubly important with patients wearing artificial teeth to see that the lips make a good seal over the outside of the rubber mouthpiece. It is a mistake to remove the teeth, for this may allow the mouthpiece to fall inward, away from the lips.

With patients with few or no front teeth, the problem is apt to become more difficult. This problem is solved, however, by having a good seal made by the lips. It may be necessary to draw the lips over the flange of the mouthpiece and hold them in place with adhesive tape.

Ruptured Eardrums.—If the patient has a ruptured eardrum and a patent auditory (eustachian) tube, air is likely to escape through the ear. If this occurs, the external ear canal is sealed with a cotton swab impregnated with petrolatum. The operator may have to hold the plug in place while the patient is being tested for leaks at the nose, for otherwise the plug may be blown out.

Making the Test.—With the patient ready and breathing room air comfortably, start the record by turning on the motor switch. The stylus should now be tracing a horizontal line near the top of the record. The actual test is started by flipping the breathing valve to the ''test'' position, preferably at the end of an expiration. As soon as it is clear that the test is proceeding normally, check the seals at the patient's nose and mouth (and ears also, if necessary) with the leak tester. To do this, take it out of its glass of cold water, wipe it dry, and then hold it under the nose and at the corners of the mouth while you watch for any condensation on the polished surface of the metal. The test should not be allowed to proceed unless all leaks have been eliminated, for a leak will spoil the accuracy of the oxygen-consumption measurement.

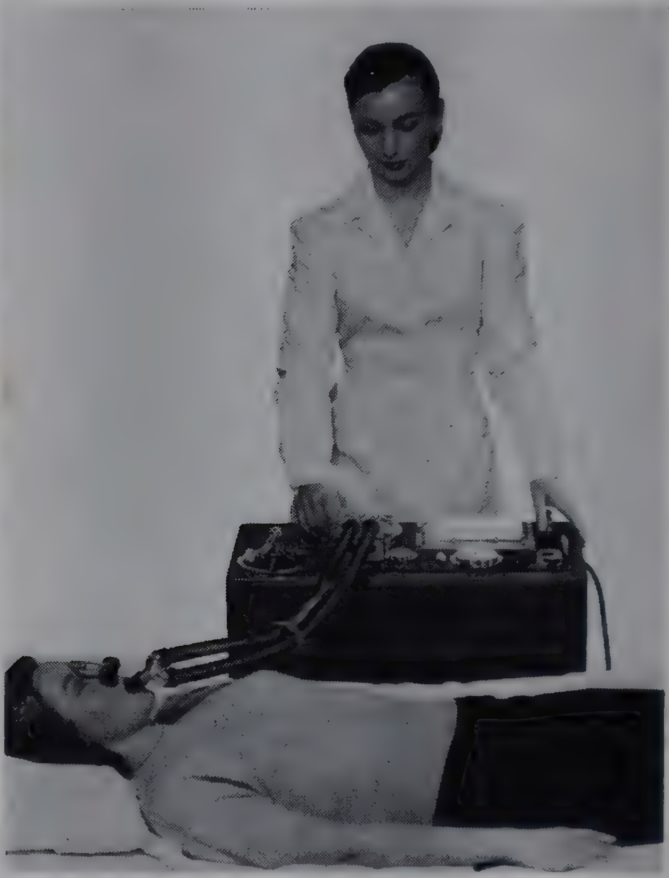
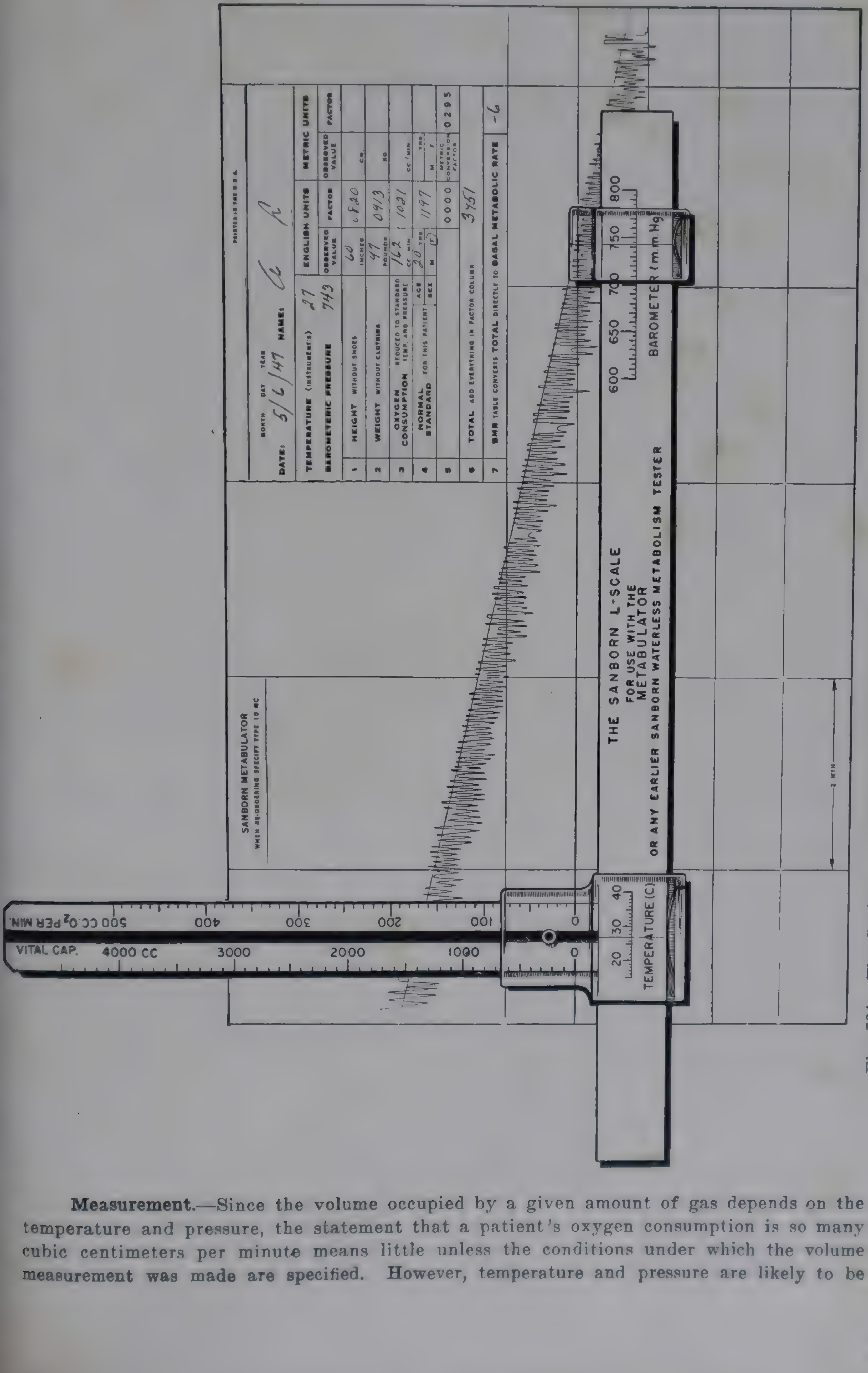


Fig. 723.—A basal metabolism test using the Sanborn instrument. (Courtesy Sanborn Company, Cambridge, Mass.)

Run the test six minutes or more unless the bellows become collapsed earlier. If a duplicate test is desired, turn the breathing valve to the ''fill'' position at the end of the first test, readmit oxygen from the tank as before, and when the bellows has been adequately filled close the valve on the tank and return the breathing valve to the ''test'' position. If the patient shows any signs of discomfort or reluctance at the end of the first test, however, remove the mouthpiece and noseclip and allow him to rest before making a second run.

At the middle of each test record the *temperature,* as read from the instrument's dial thermometer, and the *barometric pressure,* directly on the record. Tap the barometer gently before making the reading. With the test completed, throw the breathing valve to the ''fill'' position again and disconnect the patient. Pull carefully on the protruding paper in order to bring the remainder of the record out of the machine, then bend the paper sharply back over the barometer dial and move the free end a little to one side, whereupon a quick pull will tear off the protruding portion of the paper. Be sure the motor switch is turned off. Write the date, and the patient's name, height, weight, age, and sex on the record, and also the temperature and barometer readings if this has not already been done.



SANBORN METABULATOR
WHEN RE-ORDERING SPECIFY TYPE 10 MC

PRINTED IN THE U.S.A.

DATE: MONTH DAY YEAR 5/6/47 NAME: C K

TEMPERATURE (INSTRUMENT'S) 27

BAROMETRIC PRESSURE 743

		ENGLISH UNITS		METRIC UNITS	
		OBSERVED VALUE	FACTOR	OBSERVED VALUE	FACTOR
1	HEIGHT WITHOUT SHOES	60 INCHES	.820	CM.	
2	WEIGHT WITHOUT CLOTHING	97 POUNDS	0913	KG	
3	OXYGEN CONSUMPTION REDUCED TO STANDARD TEMP. AND PRESSURE	162 CC MIN	1021	CC/MIN	
4	NORMAL STANDARD FOR THIS PATIENT AGE SEX	20 YRS M [✓ F]	1197	YRS M F	
5			0000	METRIC CONVERSION FACTOR	0295
6	TOTAL ADD EVERYTHING IN FACTOR COLUMN		3451		
7	BMR TABLE CONVERTS TOTAL DIRECTLY TO BASAL METABOLIC RATE				-6

THE SANBORN L-SCALE
FOR USE WITH THE METABULATOR
OR ANY EARLIER SANBORN WATERLESS METABOLISM TESTER
600 650 700 750 800
BAROMETER (mm Hg)
20 30 40
TEMPERATURE (C)
500 CC.O2 PER MIN.
400
300
200
100
0
VITAL CAP. 4000 CC
3000
2000
1000
0
2 MIN

Fig. 724.—The Sanborn "L-scale" for use with the Metabulator. (Courtesy Sanborn Co., Cambridge, Mass.)

Measurement.—Since the volume occupied by a given amount of gas depends on the temperature and pressure, the statement that a patient's oxygen consumption is so many cubic centimeters per minute means little unless the conditions under which the volume measurement was made are specified. However, temperature and pressure are likely to be different in each test, so that it has become accepted practice to refer all gas-volume measurements to ''standard temperature and pressure,'' or ''STP'' for short. Standard temperature is 0° C. or 32° F., standard pressure is 1 atmosphere, or 760 mm. of mercury. The actual volume measurement is made under the conditions prevailing during the experiment, and then the operator calculates, using the gas laws, the volume that *would have been found* if the measurement had been made under STP conditions. This is what is meant by an oxygen-consumption rate as ''x c.c. per minute, STP.''

A measuring scale has been provided that eliminates the STP calculation.

The purpose of this test is to determine the patient's rate of oxygen consumption, expressed in cubic centimeters per minute at standard temperature and pressure. This is done by estimating the *average downward trend* of the patient's respiratory tracing. To do this, draw a ''slope line'' through the top peaks of the record. The top peaks are chosen because they represent the expiratory position of the patient's lungs, which is usually a little more regular than the inspiratory position. The slope line can be drawn through the top, bottom, or the middle of the record as long as it represents the average downward trend. If the record is regular, the slope line can easily be drawn through the top peaks. If the record is jagged, the operator must exercise some judgment in deciding what represents the average slope. If the respiratory tracing is very irregular, it is useless to attempt to place a slope line, and the test must be repeated.

Lay the record on a flat surface and, with a long ruler and a dry pen, pencil, or other scratching device, draw a straight slope line to represent the average downward trend of the tracing. Extend this slope line to the edges of the record.

L-scale.—A measuring device has been designed to enable the operator to read the oxygen consumption, in STP per minute, directly from the record. The L-scale will be found among the accessories in the case with the ''Slide-Rule Calculator.'' Hold the long arm of the ''L'' horizontal, with the lettering right-side up, and slide the shorter arm (pointing upwards) over the left-hand end of the long one. The small slider will already be in place at the right-hand end of the arm. The scale now consists of a horizontal bar carrying two sliders, each with a vertical hairline lying over a correction scale (see Fig. 724). One of these scales is for temperature and the other for barometric pressure. Adjust the sliders so that the hairlines mark the temperature and barometer readings taken during the test. Note that each slider also carries a *horizontal* hairline, lying a little above the upper edge of the long arm of the ''L.'' The line on the right-hand slider has a vertical hairline crossing it, and the L-scale is to be applied to the respiratory tracing in such a manner that the intersection of these two hairlines on the slider lies exactly over the intersection of the previously drawn slope line and one or other of the horizontal lines printed on the record (see Fig. 724). When this has been done, adjust the ''levelling'' of the ''L'' until the horizontal hairline on the *left-hand* slider also coincides with the horizontal line which has been chosen. Check the position of the intersection on the right-hand slider again, to be sure that it has not moved, and then read the oxygen consumption directly at the point where the slope line strikes the right-hand margin of the vertical arm of the ''L.'' The reading on this latter scale is the patient's oxygen consumption in cubic centimeters per minute, reduced to standard temperature and pressure.

T-scale.—While this new L-scale represents a considerable improvement over the T-scale that has been supplied with the Sanborn Waterless Metabolism Tester, it is perfectly permissible for any operator who wishes to do so to use the T-scale on the records produced by the present machine. It is applied to the tracing exactly as in the case of the waterless testers, using a vertical line for the alignment and reading the oxygen consumption along the horizontal arm. Corrections for the temperature and pressure are made, of course, by adjustment of the sliders before the scale is applied.

Conversely the L-scale may be used to measure a slope line produced on one of the older waterless machines. Since alignment of the L-scale depends on a *horizontal* line, it will be necessary to draw in a base-line on the Waterless chart, which can be done most conveniently before the chart is removed from the machine. Simply inflate the bellows to the point where the writing stylus will cross the respiratory record near its end and then, maintaining the bellows at this volume, give the drum one full turn by hand. This will provide a horizontal base-line for the L-scale. If it has been placed correctly, as suggested above, the base-line and the slope line will cross near the right-hand edge of the record. If the base-line is too high or too low, it can easily be drawn in a more appropriately placed line parallel to the original.

Basal Metabolic Rate.—The Metabulator will provide the operator with a measurement of the patient's oxygen consumption in STP c.c. per minute. The BMR goes a step further—it is a measure of how nearly normal the determined oxygen consumption is for the particular patient concerned, taking his height, weight, age, and sex into account. The BMR is expressed as a figure (with a prefixed plus or minus sign) showing the *percentage* by which the patient's oxygen consumption is above or below normal. Thus a BMR of +42 indicates an oxygen consumption 42 per cent too high; while -17 indicates one 17 per cent below normal, etc. The problem now is how to convert the figure found for oxygen consumption into a BMR for the patient concerned, thus telling by how many percentage points the oxygen consumption differs from the normal.

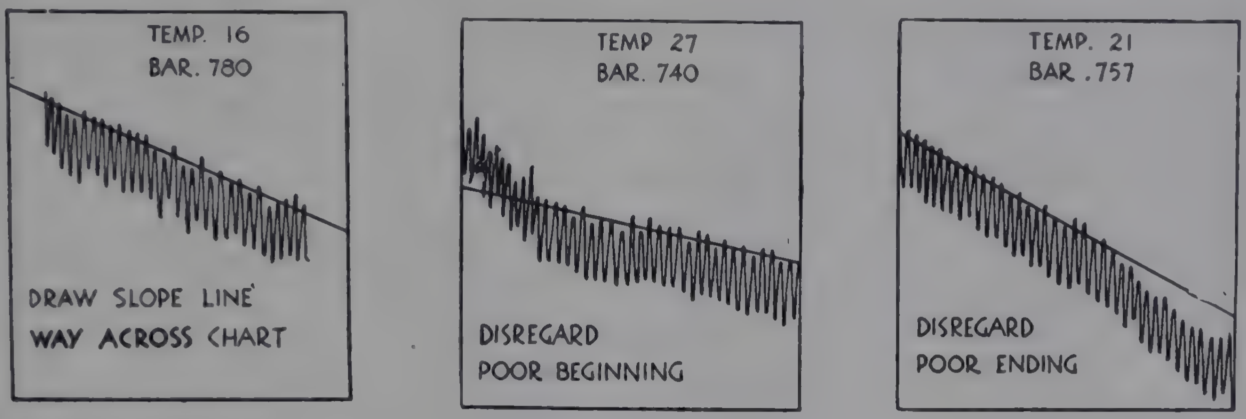


Fig. 725*A*.—How to draw slope lines, Sanborn basal metabolism test. (Courtesy Sanborn Co., Cambridge.)

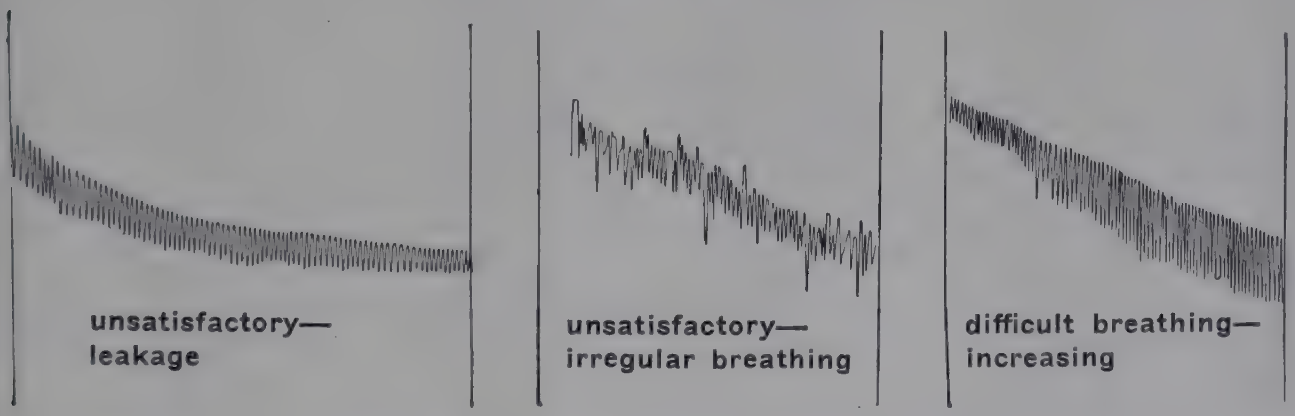


Fig. 725*B*.—Types of slopes that are to be discarded in making Sanborn basal metabolism tests. (Courtesy Sanborn Co., Cambridge.)

This conversion may be accomplished either by the "Slide-Rule Calculator" supplied with the accessories or by using metabolism tables. The former is more rapid; the latter more accurate. The metabolism tables also cover more extreme cases, and can be used with either English or metric units for height and weight.

Slide-Rule Calculator.—Set the hairline on the slider to the figure found for oxygen consumption.

Move the central scale until the male or female age, as the case may be, lies under the hairline.

Move the hairline again until it lies over the correct height.

Slide the central scale until the weight lies under the hairline.

Finally read the BMR opposite the black arrow at the top edge of the central scale.

Metabolism Tables.—The tables formerly used with Sanborn metabolism testers are perfectly adequate to convert oxygen consumption to BMR. The new set of tables will greatly facilitate the calculation involved. The printing on the recording paper is designed so that the whole computation can be carried out on the original record.

The quantities concerned in the calculations are: (1) the patient's height, (2) his weight, (3) his rate of oxygen consumption (L-scale reading), (4) his age and sex.

The newer tables give a factor for each of these four measurements, and a column is provided on the chart for the factors found, so that they can be put down one above the other. They are then added together and the sum is converted directly to a BMR through the use of a final conversion table.

English or Metric Units.—The principle involved in this method of calculation makes it possible to use the same tables whether the heights and weights are in pounds and inches on the one hand or in kilograms and centimeters on the other. The difference is taken care of by the addition of a ''metric conversion factor'' if the metric units are used. Examine the printed box on one of the charts. The tabulations are set up so that if the bodily measurements are taken in English units the left-hand section is used, with no conversion factor; whereas if the measurements are in centimeters and kilograms the right-hand column is used, and the metric conversion factor is printed at the bottom of the factor column so that it will be added in along with the other factors. The sum in line 6, obtained by the addition of all the figures in the factor column, will be the same whether the left-hand section with English units is used, with no conversion factor to add in, or whether the right-hand section with metric units is used, and the conversion factor ''0295'' added in. Since the sum in line 6 is the same for either system of units, the BMR in line 7 will also be the same, as it should be.

Height Factor.—Measure the patient's height to the nearest inch or centimeter. In the column at the left-hand side of Table 1 on the recording paper will be found the heights arranged in steps of ten. Suppose the patient's height is 67 inches. Since 67 = 60 + 7, follow the left-hand column down to ''60,'' then follow that horizontal line across to the column headed ''+7.'' This factor, 0750, is the correct one for a height of 67 inches. Other values will be treated in exactly similar fashion. If the height is 182 centimeters, follow the height column down to ''180'' and then follow the horizontal line across to the column headed ''+2,'' where the factor 0121 will be found, etc.

Weight Factor.—Measure the patient's weight without clothing in pounds or kilograms.* Find the weight factor in Table 2 on the recording paper. For example, if the patient's weight is 145 lbs., follow the weight column down to ''140'' and then trace that horizontal line over to the ''+5'' column, where the factor 0765 will be found.

Oxygen-Consumption Factor.—Use Table 3 on the recording paper to find the factor for oxygen consumption. The procedure is the same as for the other two. The figure to start with is the number of STP c.c. of oxygen per minute as determined from measurements of the patient's record. For a consumption rate of 217 c.c. per minute, for example, find ''210'' in the left-hand column and then follow that line across to the column headed ''+7.'' The factor is 1275.

Normal Standard Factor.—Since the basal metabolic rate depends on a comparison of what the patient actually does with what a normal person of his age and sex ought to be doing, a factor must be found representing ''normal'' behavior. Table 4, showing the factors for the normal standard, will be found on the back of the tables card. The column for the age is in the middle, and the factors for male patients are found at the left, while those for females are to the right. Some of the factors are the same for a number of different ages. This is all right, for that is the way normal people behave. The groupings also differ between males and females.

Find the patient's ''normal standard'' factor and put it down in line 4; e.g. for a female patient, aged 29, it would be 1208. For a male of the same age, it would be 1114.

Conversion to BMR.—When all the factors have been added (including the metric conversion factor if the height and weight were in centimeters and kilograms) the total is converted directly to BMR through the use of Table 5 on the recording paper. Reduce the sum to three figures by discarding the fourth, increasing the third figure by 1 if the fourth is 5 or more. Thus, if the total is 4316, call it ''432''; while if it is 4312 it becomes ''431.'' Using the conversion table just as the others were used, it will be seen that ''431'' gives a BMR of +43. Insert the result in line 7 on the chart.

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*Needless to say, heights and weights must be either *both* English or *both* metric; do not try to mix a metric height with an English weight, or vice versa.

JONES METHOD

This method was devised by Dr. Horry M. Jones, of Chicago. The machine is a portable instrument, consisting of a bellows mounted on a strong metallic stand with a standard liter measuring device which automatically corrects its oxygen volume with the barometer and thermometer without the use of a barometer and thermometer.

The metabolism test is a quantitative determination, and therefore requires more than ordinary precision in technic. Results should be within from 1 to 5 per cent of the patient's true metabolic rate, otherwise the technic is being very crudely performed.

Set up the apparatus, as illustrated. Carry out each step in the exact sequence as given.

Connect oxygen tank in place using wheel wrench to tighten it against leather gasket. Place the tracing sheet on the clock plate with the lettered end of the clock plate up.

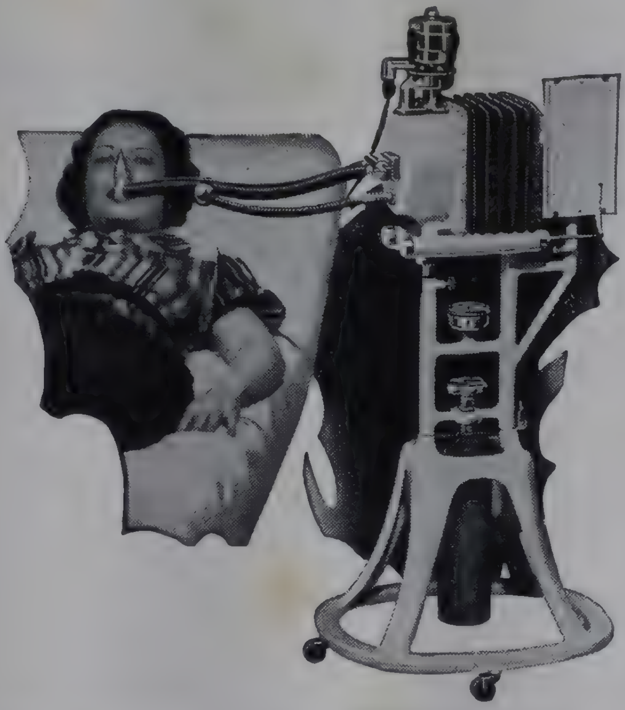


Fig. 726.—The Jones basal metabolism apparatus. (Courtesy Middlewest Instrument Co., Chicago.)

Adjust clock plate so that its geared bar fits in the slot of clock housing and then slide plate down into slot until it comes to rest against geared wheel inside of housing. If the clock starts, stop it by snapping switch to OFF position. If it does not start with switch in ON position, lift clock plate about ½ inch and bring it down with slight downward jerk. Then see if the clock can now be started by turning on switch. If this has succeeded in getting geared bar and geared wheel to mesh, stop clock until it is to be started as directed later.

Keep the pen tip free of dried ink or lint by drawing a pinpoint through it so that the pen will write with a fine, sharp line. Fasten the pen to its bar and rotate the clock in its seat until the pen traces evenly across the entire width of the clock-plate. The mouthpiece (disinfected, not by boiling, but by briefly immersing into wide-mouthed jar of 50 per cent alcohol, and then rinsed with water) is now attached to the breathing tubes, so rotated that it faces the patient's mouth squarely without twisting the tubes.

Stand the apparatus at exactly the correct distance (move apparatus to patient, not the patient to the apparatus) so that the mouthpiece will just reach the patient's mouth without bending the breathing tube.

Turn the three-way valve so that its pin is parallel to the floor. Pull on the bellows until the point of the pen is at the air position on the graph. Holding the bellows in that position, turn the three-way valve perpendicular to the floor, and keep the bellows at the air position.

Close the needle valve. This retains the oxygen in the measuring chamber.

Admit an unknown amount of oxygen into the bellows. The black needle on the oxygen gauge below the bellows should point around 10° to 20°.

Open the needle valve and admit enough of this oxygen into the bellows to push the pen point to S, M, or L on the paper (Small, Medium, or Large, depending on the size of the patient.)

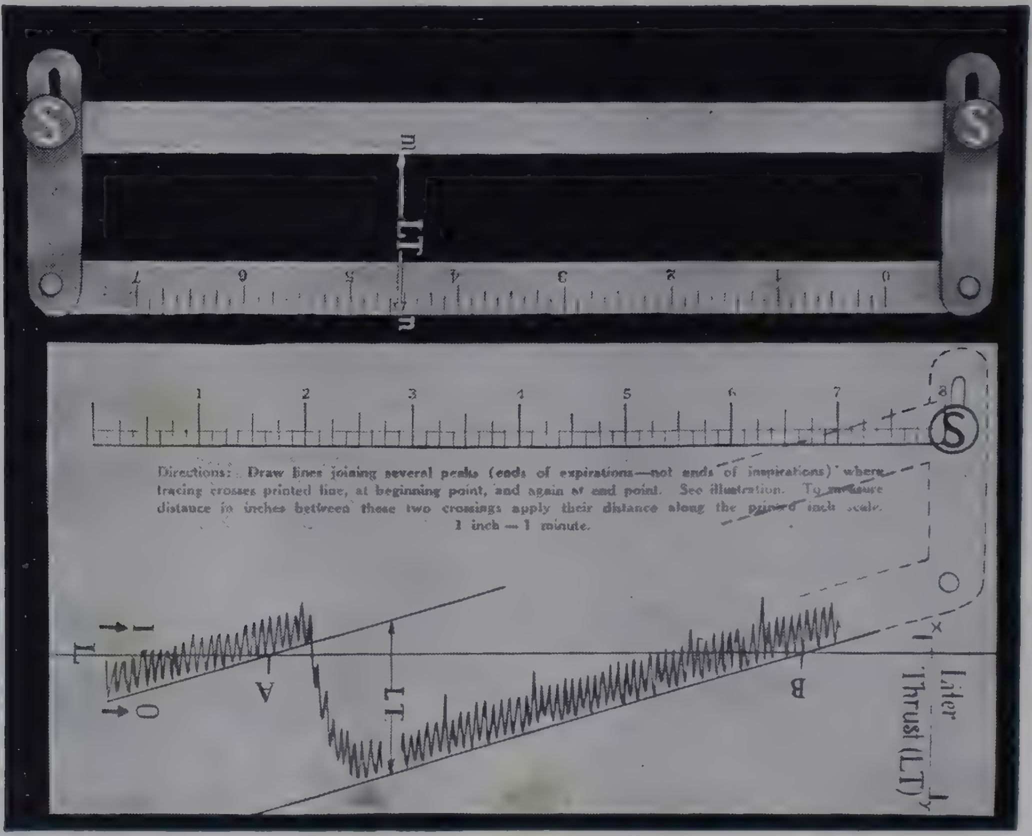
Admit an exact liter of oxygen into the measuring chamber by turning the wheel wrench on the oxygen tank until the black needle is superimposed on the red needle of the gauge.

Attach the nosepiece to the patient. Connect the mouthpiece to the apparatus. Instruct the patient how to use the mouthpiece, and insert it into the patient's mouth.

Start the motor of the apparatus.

At the extreme end of the patient's expiration turn the three-way valve parallel to the floor, thus connecting the patient to an unknown quantity of oxygen in the bellows.

After a few respirations, start the clock, and make the pen write on the graph.



Directions: Draw lines joining several peaks (ends of expirations—not ends of inspirations) where tracing crosses printed line, at beginning point, and again at end point. See illustration. To measure distance in inches between these two crossings apply their distance along the printed inch scale. 1 inch — 1 minute.

Fig. 727.—Jones basal metabolism protractor and sample test chart. (Courtesy Middlewest Instrument Co., Chicago.)

When the tracing has crossed the base line at A (receded within the black line on the paper), open the needle valve and admit the known liter of oxygen from the measuring chamber into the bellows. This will push the pen out beyond the black line. When the pen recedes within the black line again at B, the test is ended.

Testing for Nose Leak.—After the noseclip has been placed in position, instruct the patient to try to breathe through his nose. If he is able to breathe through his nose while the clip is in place, adjust it to make it airtight. If there is a nose leak, the tracing will be concave instead of a straight line slope.

Testing for Mouth Leaks.—If there is a mouth leak due to improper adjustment of the mouthpiece, the tracing will be a back and forth movement of the pen instead of a slope.

Calculation of Basal Metabolic Rate.—Use the protractor to determine the time required to consume one liter of oxygen, as follows: Apply the top edge of the top ruler of the adjusted protractor along the long or "liter" slope of the tracing on the paper to line up with the highest peaks. Do not allow more than one or two peaks to project above the ruler edge. Holding the upper rule in this position, swing the connecting arms to such position that either edge of the connecting arm lies PARALLEL to the edge of either end of the

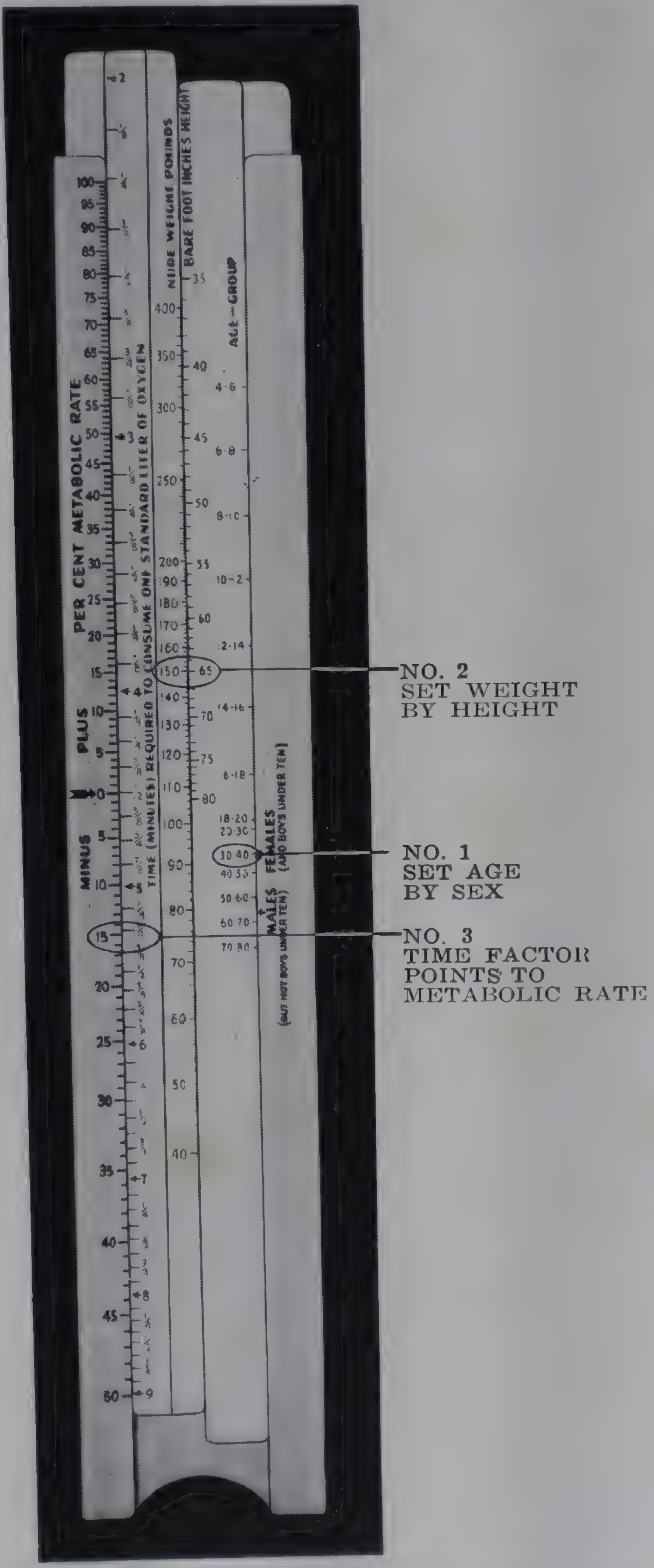

PER CENT METABOLIC RATE
PLUS
MINUS
TIME (MINUTES) REQUIRED TO CONSUME ONE STANDARD LITER OF OXYGEN
NUDE WEIGHT POUNDS
BARE FOOT INCHES HEIGHT
AGE—GROUP
FEMALES (AND BOYS UNDER TEN)
MALES (BUT NOT BOYS UNDER TEN)
NO. 2
SET WEIGHT
BY HEIGHT
NO. 1
SET AGE
BY SEX
NO. 3
TIME FACTOR
POINTS TO
METABOLIC RATE

Fig. 728.—Jones basal metabolism calculator. (Courtesy Middlewest Instrument Co., Chicago.)

tracing sheet. The shorter slope peaks should now line up with the upper edge of the lower ruler. If they do not, it indicates (1) that a full liter was not admitted to the breathing chamber; or (2) that part of it was lost through a nose leak or mouth leak; or (3) that soda lime has been used for more than a hundred tests; or (4) that a full minute preliminary breathing period was not given; or (5) that the patient was not made to relax for even breathing by taking a full, deep inspiration.

Draw a line along the top of the two edges of the protractor. A is the point where the shorter, or first slope crosses the printed line; B is the crossing of the longer slope. The distance between them, in inches, is the time in minutes required by the patient to consume the standard liter of oxygen.

Obtain the age of the patient, his weight without clothing, height without shoes, and sex.

Read off the basal metabolism rate on the automatic calculator.

Determine the Liter Thrust of the Pen.—When a liter of oxygen is discharged into the bellows, the pen moves outward by the amount of the expansion of the bellows. This we call the liter thrust of the pen. Measure up a liter in the usual way. Do not have the apparatus connected to a patient for this part of the procedure. Next open the three-way valve and pull the bellows carried by the hand to a point such that the pen tip is about ¾ inch inside of the printed line of the tracing sheet, then close three-way valve to hold the bellows at that point. Let the clock-plate run to trace a short line (X) to show this position of the pen tip. Stop the clock-plate. Discharge the liter of oxygen now in the measuring chamber, making sure the indicator swings from the room temperature point on the gauge scale backward all the way to within ⅛ inch of the self-verifying zero of the gauge. The self-verifying zero is a short, black calibration mark somewhere near and slightly above the word ''caution'' at the edge of the gauge dial. If the indicator does not make this full swing, you have not delivered a liter of oxygen to the bellows. After all of the oxygen has gone into the bellows, that is, after the pen has been thrust out as far as this liter pushes it, again let the clock run to trace another short line (Y) to show its new position. The distance between (X) and (Y) is the ''liter thrust.'' This part of the procedure should be done independently of the test, that is, any time after or before the test. It is done only for the purpose of adjusting the rulers of the protractors and occasionally as a check-up to see if the protractor rulers are still adjusted to equal the distance of the liter thrust.

To adjust the rulers of the protractor to fit this liter thrust, release the knurled nuts of the short arms of the protractor, then move the rulers closer in, or farther apart, until the upper edge of one ruler coincides with (X) and the upper edge of the other ruler coincides with (Y). Adjust both ends of the protractor so that the upper edges of rulers equal the distance from (X) to (Y) throughout the entire length of the protractor, then tighten the knurled nuts to hold the rulers to that adjustment. At sea level and 25° C., this liter thrust of the pen is approximately 1½ inches, and for each thousand feet of elevation above sea level, this liter thrust is increased about 1/12 inch. For example: At about 6,000 feet above sea level, the liter thrust would be 1½ inches plus 6/12, or 2 inches. This serves as a means of determining whether or not you are actually delivering the standard liter regardless of the barometric pressure changes caused by changes in elevation above sea level.

Having determined the liter thrust of your machine for the sea-level elevation of your locality, this thrust is approximately constant, and thereafter it is unnecessary to adjust the rules of the protractor to equal the liter thrust, except as an occasional check-up.

THE JONES DOUBLE BELLOWS MOTOR-BASAL

This apparatus differs from the Jones MOTOR-BASAL in that there are two bellows instead of one (one inside the other). The larger of the two bellows has exactly twice the cross-section area of the smaller bellows.

By means of a conversion valve, a single turn converts the apparatus to either a small bellows or a large bellows hook-up.

The smaller bellows hook-up is used on a small sized patient, who would be connected to the apparatus for an unnecessarily long period if a large capacity bellows were used. The large bellows hook-up is used on a large sized patient who would be connected to the apparatus a relatively short time if a small bellows were used. Thus, the capacity of the apparatus is adjustable to the size of the patient.

CHAPTER XVII

ELECTROCARDIOGRAPHY

By Julius Elson, M.D.*

The frequency with which disorders of the cardiovascular system are encountered in the practice of medicine and the importance of the electrocardiograph in the diagnosis, treatment, and prognosis of these disturbances has made it desirable to include a discussion of this important subject in this book.

It is not within the scope of this chapter to undertake an explanation of the theories underlying electrocardiographic interpretation and all the patterns which present themselves in clinical practice. These subjects are covered thoroughly in standard texts devoted solely to the subject of electrocardiography. This chapter will present the principle and the use of the electrocardiograph, and will point out the more important patterns, clinical applications, and pitfalls of electrocardiography.

THE USE OF THE ELECTROCARDIOGRAPH

Because the terms *electrocardiograph* and *electrocardiogram* are so often confused, it is well to point out that *electrocardiograph* refers to the recording instrument, whereas *electrocardiogram* refers to the record itself. The electrocardiograph is an instrument by means of which a visible record is made of the many minute electrical or electrochemical changes occurring in the *heart muscle* associated with its contraction. It is important to stress the point that the electrocardiogram is a reflection merely of electrical activity of heart *muscle* so that one will recognize the limitations of the clinical application of this means of diagnosis.

For instance, in valvular disease, or in certain congenital malformations where the heart muscle is not primarily involved, the electrocardiogram cannot be expected to be of help except in those cases where secondary heart muscle changes occur, usually in the form of hypertrophy of the auricular or ventricular musculature. These secondary effects on the musculature give a clue to the primary valvular disease; but until these effects do occur the electrocardiogram is often normal in the presence of valvular disease.

The electrocardiogram may be normal even in the presence of myocardial involvement if the site of the lesion is not in the proper axis with regard to the electrocardiographic "leads."

All laboratory tests must be interpreted in the light of the clinical picture. Laboratory tests alone should rarely if ever be relied upon for making a diagnosis or determining treatment and prognosis. This is an extremely important point, and must constantly be borne in mind. In the experience of many electrocardiographers, patients are often referred for electrocardiographic tracings in the belief that such tracings may give the answer to the whole question of diagnosis. Actually, so many factors can affect electrical action currents in the heart that even experienced electrocardiographers may not be able to make proper electrocardiographic diagnoses without ample clinical data. Even with this information it may be impossible to make a decision in borderline electrocardiograms because of the many variations of the normal electrocardiogram.

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While a normal electrocardiogram does not necessarily exclude the presence of cardiac disease, an abnormal tracing surely indicates some disorder. This disorder may be organic in nature, or it may involve disturbances in the cardiac mechanism; or it may merely reflect an abnormal psychosomatic effect on the heart. Many extracardiac influences can alter the electrocardiogram. Toxic substances, either bacterial or nonbacterial, and disturbances in other organs which might affect the position of the heart in the thoracic cavity, are among the factors which sometimes alter the tracing. Drinking cold water, emotional disturbances, and smoking may also alter the normal electrocardiographic pattern.

VALUE OF THE ELECTROCARDIOGRAPH

The greatest value of the electrocardiograph lies in the field of coronary artery disease in which the myocardium is involved acutely or by progressive degenerative changes. The electrocardiograph in these conditions is of great value since in many instances there are no objective positive clinical findings.

Often in acute myocardial infarction the electrocardiogram will present a pathognomonic picture, especially if serial electrocardiograms are taken and these electrocardiograms are found to undergo characteristic changing patterns. While acute myocardial infarction without significant electrocardiographic abnormality does occur, such a diagnosis should be made with reservation. Although the diagnosis can often be made clinically, it is a great comfort to the clinician to have the added assurance of the electrocardiographic diagnosis. This may be of vital importance to the patient also, since on occasion the symptoms may be confusing and may simulate those of an acute surgical abdominal condition or dissecting aneurysm. Patients have been operated upon for perforated peptic ulcer, when in reality they were suffering from myocardial infarction; others have been spared needless surgery by making an electrocardiogram which disclosed the cardiac nature of the patient's symptoms.

In addition to its value in coronary artery heart disease, electrocardiography is very useful in the positive recognition of certain cardiac arrhythmias and disturbances of conduction, the exact nature of which could not be ascertained without recourse to cumbersome methods of recording venous pressures, arterial pulses, and the apex impulse. The electrocardiograph has made the certain identification of these abnormal mechanisms a simple matter.

This instrument can sometimes be of aid as a guide to treatment. Recognition of characteristic alterations of certain segments of the tracings or of clinically obscure disturbances in rhythm and conduction which have occurred in the course of treatment with certain drugs, most commonly digitalis, is often the signal for discontinuation or more cautious administration of such drugs.

From a prognostic standpoint, conclusions from an electrocardiogram alone are most precarious. Prognosis should be made from an evaluation of the clinical aspects of the case. For instance, the presence or absence of heart failure, which is not indicated by the electrocardiogram, is very important. If such disturbances as complete heart block or bundle branch block are found in the tracing, the prognosis is usually poor; not infrequently, however, a patient with bundle branch block may live for a number of years. A patient with myocardial infarction may, on the other hand, show an electrocardiogram approaching a normal pattern, yet he may die of cardiac rupture, embolism, ventricular fibrillation, or heart failure.

The characteristic alterations of the electrocardiogram caused by digitalis have led to another field of usefulness for the electrocardiograph; namely, in the standardization of digitalis glycosides, using human subjects.

PRINCIPLE OF ELECTROCARDIOGRAPHY

The science of electrocardiography was first made possible in the middle of the 19th century when it was discovered that the contraction of the frog's heart was accompanied by an electric current. By connecting the outside surface of the human subject by means of electrodes to a capillary electrometer, Ludwig and Waller in 1887 demonstrated that the electrical currents in the heart could be recorded. Because of the nature of the instrument, records at that time were inaccurate, and the method was not practicable. It was not until 1903, when Einthoven applied his string galvanometer to the study of the human heart, that electrocardiography was put on a firm, clinically reliable basis. Only with an understanding of the electrocardiogram in electrical terms can one appreciate the magnitude of Einthoven's contribution to medicine.

The electrocardiogram, as recorded by electrodes placed upon the body surface, consists of a series of impulses, each of which is of relatively short duration. The baseline above and below which these impulses fluctuate is known as the *isoelectric level.* Some of these impulses may rise from the isoelectric level to their peak in a time interval as short as 0.02 second while the amplitude may represent as little as one millivolt.

Furthermore, the resistance of the circuit within the patient, the *patient circuit,* as measured between a pair of electrodes, averages under good conditions about 2,000 ohms. On the basis of one millivolt amplitude and 2,000 ohms resistance offered by the patient, the peak power which the body generating the electrical impulses can deliver to the recording instrument would be only one-eighth of one-thousandth of one-millionth of a watt.

String Galvanometer.—The string galvanometer is based upon the fundamental principle that a conductor which carries an electric current, and which is suspended in a magnetic field, will tend to be deflected from its original position by a force acting on that conductor. This force is produced by the reaction between the current and the magnetic field.

String Shadow.—In the usual electrocardiograph the current-carrying conductor is a quartz "string" about one five thousandth of an inch in diameter (5 μ), covered with a metal such as platinum. This string is suspended between the north and south poles of an electromagnet. Without stretching this platinum-coated quartz string very tightly one may time this light fiber to a sufficiently high natural frequency of vibration to produce a moving system capable of following the details of the human electrocardiogram with a satisfactory degree of accuracy.

The actual motion of the "string" through which the current from the patient passes is so small that it must be magnified from 500 to 1,000 times. This is accomplished by means of a microscope through which light is thrown by an electrical source and condenser. The magnified shadow of the "string," with all its movements, is thus projected on a camera. The recording medium, usually bromide paper, is pulled through the camera at a constant speed. A timing device is provided by a spoked wheel rotating in the path of light and periodically interrupting the light to produce timing coordinates, thus allowing measurement of the various events of the cardiac cycle.

With most timers using a rotating wheel, the fifth spoke is wider, so that every fifth vertical time line is broader than the other four. The distance between each fine line represents 0.04 second; that between each wide line, therefore, represents 0.20 second.

A piece of glass with parallel lines ruled on it is mounted just in front of the paper so that it casts fine horizontal shadows on the sensitized paper at one-millimeter intervals. The resulting horizontal lines are the coordinates by means of which the amplitude of the electrical deviations is measured.

This combination of the string shadow, the light interruptions produced by the timer, and the ruled scale for the amplitude coordinates results in a completed record in which the string movements and the time and amplitude coordinates appear as white lines on a dark background.

Leads.—There are three main types of leads in general use today: (1) the bipolar or conventional leads, (2) the unipolar leads, and (3) the augmented unipolar leads.

Bipolar leads are obtained by placing metal electrodes on two different points of the body. By convention, and because experience has justified it, these leads are recorded from the right arm, left arm, and left leg, as well as from various positions on the chest.

The lead taken from the right and the left arm is known as **Lead I**; that from the right arm and the left leg is called **Lead II**; and that from the left arm and left leg is called **Lead III**. These are the conventional bipolar leads universally used.

The chest leads are designated in a variety of ways, depending on the position of the chest electrode, or *exploring electrode*, and the limb electrode, or *indifferent electrode*. For further clarification of certain complexes of the electrocardiogram even esophageal leads have been used; in these a specially designed electrode is passed down into the esophagus and electrocardiograms are taken with this electrode held at various levels.

In recent years, the method of unipolar lead electrocardiography has been widely adopted. These leads more nearly record the absolute potential at each of the extremities as well as at the exploring electrode on the chest wall.

Unipolar Leads.—There are two types of unipolar leads: (1) the Wilson type, and (2) the augmented unipolar limb leads. Unipolar leads are described on pages 2407 ff.

The use of ''unipolar limb leads'' has become general. The connections are made as for the ''central terminal'' (V) leads of Wilson, described on page 2405. Three records are then made, with the precordial electrode placed on the right arm, on the left arm, and on the left leg, respectively. A discussion of the theoretical considerations and clinical applications of the unipolar leads can be found in *Unipolar Lead Electrocardiography* by Emanuel Goldberger* and many other publications devoted to this subject.

The choice of the lead or electrode pair to be recorded is made by a *lead selector* switch on the panel of the instrument.

Other Currents.—In addition to the currents caused by the action of the heart there are other currents, such as those arising in the skin, which cause deflections in the string. These currents are relatively constant, but unless counteracted they will produce a movement of the string away from the center or zero position which will make the recording of the heart action current impossible.

To compensate for, or to neutralize the effects of these currents, there is incorporated in the electrocardiograph an ordinary dry cell such as is used in a simple flashlight. This necessitates another switch, the *compensating switch,* which is adjusted in a manner to keep the string centered despite the fluctuations of the extracardiac currents.

Standard Records.—Records made at different times, or by different instruments, in order to be comparable, must record the same electrical quantity according to the same scale. The unit of measurement is the millivolt, and the scale of recording is one centimeter, which corresponds to one millivolt developed at the electrodes.

To standardize an electrocardiograph, the sensitivity of the string is so adjusted that it responds to a one millivolt current by deviating one centimeter from the base line or zero level. The one millivolt standardizing current is introduced from a source built into the instrument. If the sensitivity of the string is such that it deflects more than one centimeter, the tension of the string must be increased; that is, the string must be tightened by means of a screw attachment until the deflection or ''jump'' of the string is exactly one centimeter. If the sensitivity is such that the string ''jump'' is less than one centimeter, the string is then accordingly loosened by the screw attachment.

Before standardizing a string galvanometer type of electrocardiograph it is necessary that the patient, the galvanometer, and the standardizing circuit all be connected. Resistance in the patient circuit varies from time to time and from lead to lead; for this reason it is necessary to restandardize the instrument for each lead while the string and patient circuit are connected and the string is responding to cardiac action currents.

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*Goldberger, Emanuel: Unipolar Lead Electrocardiography, Philadelphia, 1949, Lea & Febiger.

Skin Resistance.—The patient circuit resistance derived from the skin must be kept low, two thousand ohms or less. Too high resistance necessitates ''loosening'' the string to such an extent, in order to make it jump one centimeter, that ''overshooting'' of the string results. ''Overshooting,'' or string fling, results in distortions in the tracings which make it difficult or even impossible to evaluate the electrocardiogram properly.

Skin resistance is lowered by the application of some form of electrode paste or jelly to the skin at the site of the electrode. This paste contains an electrolyte; when rubbed into the skin with a little excess on the electrode, it reduces the resistance between the electrode and the tissues beneath the skin from a value of many thousands of ohms to about one thousand ohms.

Electron Tube Amplifier.—Another type of electrocardiograph was later developed and is now in common use. It employs the electron tube amplifier principle. This type of instrument makes use of an amplifying circuit which greatly magnifies the tiny cardiac currents, and includes a very large resistance to prevent overshooting. The most popular of this type instrument employs a very small mirror attached to the movable element or coil in the magnetic field. This mirror is so arranged that by its motion it swings either a spot of light or a field of light with a shadow in it across the face of the recording camera.

An advantage of this type of apparatus is that it is not necessary to standardize the instrument with the patient switched in. This enables the operator to make the tracing without any disturbance to the recording beam other than that caused by its response to the standardizing pulse. Still another advantage is that standardization need not be repeated after the patient is switched in, nor for each of the leads, as in the case with the string electrocardiograph. These advantages are possible because of the relatively high resistance and current in the amplifying circuit as compared with the slight resistance caused by the skin or by polarization. All of these electrocardiographs require the use of photographic paper or film for recording the electrocardiogram.

Direct Writing Electrocardiographs.—Direct writing electrocardiographs have in many places supplanted the photographic types of instruments. These apparatus, by use of high-power radio tube amplifiers, have made possible direct, immediately visible recordings of the electrocardiogram. In addition to this very real advantage, the need for photographic materials and a dark room is obviated.

Vectorcardiography

This method of electrocardiographic study is not new, but in the past few years has been studied with greatly renewed interest and emphasis. There are two ways of analyzing electrocardiograms. One is the conventional or ''pattern'' method, with which we are largely concerned in this chapter, and the other is the ''vector'' method of analysis. In the former, we learn by experience and correlation with pathologic specimens the various electrocardiographic patterns associated with disease entities, whereas in the latter the many electrical forces developing and receding in the cardiac cells are represented by an arrow, the length of which measures their resultant magnitude and the tip of which their direction at any given instant. These arrows, representing the magnitude, direction, and sense of this force at a given intent, will vary from moment to moment as the impulses travel through the heart fibers in the various directions. These "instantaneous vectors" can be constructed from the conventional electrocardiogram taken to include the three bipolar limb leads, the three unipolar limb leads, and the six unipolar chest leads. By connecting the tip of each arrow representing the vector of electrical potential from moment to moment, a "loop" is formed, graphically depicting the direction of the vectors throughout the cardiac cycle. These vector loops can be much more accurately depicted by means of an oscillograph connected with the body by means of specially placed electrodes.

This method of interpretation has the advantage of not requiring memorizing of the numerous patterns with their wide range of normality; one merely visualizes abnormalities in direction and magnitude in the electrical potentials.

There are serious disadvantages to the routine adoption of spatial vectorcardiography as a routine method of study in clinical practice. The chief of these perhaps is the impossibility at this date of determining time relationships of the various phases of the cardiac cycle, and the inability to identify accurately arrhythmias. Vectorcardiography should still be regarded as an experimental method and until the objections mentioned above are obviated, and until some standard method of lead placement is generally agreed upon, it perhaps should not be adopted as a routine method in clinical practice.

The Ballistocardiograph

While a discussion of this relatively new instrument which is being found increasingly useful in the study of cardiac dynamics is not strictly within the province of this chapter, nevertheless it is of value to at least mention in a general way the nature of this type of device and its scope.

In contrast to the electrocardiograph which is an instrument that records the *electrical* events during the heart cycle, the ballistocardiograph records the movements transmitted to the body by the mechanical effects of the contraction of the heart. These movements occur in well-recognized patterns with each heartbeat, and, magnified many times by the instrument, are recorded in repeatedly reproducible patterns.

The value of the instrument lies in the fact that, since it records the certain phases of the mechanical action of the heart, it is related to the heart's efficiency as a pump. It has been shown that the ballistocardiogram changes with age, hypertension, coronary disease, has a characteristic pattern in coarctation of the aorta, and is altered by smoking. Indeed, in some instances it will show changes indicative of coronary disease before any other methods of study can reveal them. It must not, however, be inferred from this that the opposite does not happen. There certainly are instances in which the ballistocardiogram is normal and other methods of study show unmistakable evidence of abnormality. There are many types of ballistocardiographs, none entirely satisfactory. Much work is being done to evolve an instrument that will meet all basic objections. Up to this time, not even the origin of all the waves of the lead ballistocardiogram is understood.

GENERAL DIRECTIONS FOR USING A STRING-TYPE ELECTROCARDIOGRAPH (CAMBRIDGE)

Detailed descriptions of all the electrocardiographs on the market and their step-by-step operation will not be given here, since they vary in certain respects from each other. Complete information can be found in the brochures which each manufacturer supplies with the instrument. The method of taking an electrocardiogram with a standard string electrocardiograph, the Cambridge instrument, is described below.

Preparation of the Patient.—The room temperature should not be too low, because a cold room may cause quivering of muscles which results in artefacts in the tracings. The patient should preferably not have partaken of excessively cold drinks and should not have smoked immediately prior to the taking of the tracing, since cold drinks and smoking sometimes modify the tracing. The patient is instructed to bare the forearms, the chest, and the left leg, and to lie down. The supine position is preferred to the sitting position for making tracings because alterations of the electrocardiogram may occur in some individuals in the upright position.

Preparation of the Electrodes.—Some electrode jelly is rubbed rather briskly into the skin of each forearm. The massaged areas should be somewhat larger than the size of the electrodes. Massaging into the skin should be to a point of slight erythema to minimize the effects of skin resistance.

Electrode jelly suitable for electrocardiographic purposes is made by several manufacturers. The purpose of this jelly is to lessen skin resistance, improve contact, and to conduct the cardiac current through the skin to the electrode. The basic ingredients are salt, glycerine, pumice powder, gum tragacanth, potassium bitartrate, and water. A small amount of the electrode jelly is spread over the surface of the electrode which is to be in apposition to the skin. The site of application of the electrodes may be anywhere on the arm, but for convenience the anterior surface of the forearm just above the wrist and the lateral surface of the left leg just above the ankle are used.

Care must be exerted in massaging the electrode jelly so that areas of skin disease or marked varicosities are avoided.

It makes little difference where the electrode is placed with respect to the limb leads, but it is very important that the chest electrode be placed in the exact manner prescribed. Slight changes in position are likely to cause significant effects on the electrocardiogram.

If the skin in the areas where the electrodes are to be placed is hairy, use the jelly more freely and rub it into the skin more briskly than usual.

The electrodes are strapped onto the arms and the left leg in the prepared areas. It is of utmost importance that the electrodes be in complete contact with the skin since occasionally tension on the wires by movement of the patient will cause partial separation of the electrode from the skin. If the electrode is strapped on too loosely, artefacts may be caused in the record by slipping of the electrodes; if applied too tightly, artefacts due to muscle tremor may be produced.

The standard type of chest electrode has an insulated handle which may be held in place on the chest wall by the patient or by an assistant.

Lead Wires.—The lead wires are connected to the limb electrodes, care being taken that the right arm lead wire is connected to the right arm electrode, the left arm wire is connected to the left arm electrode, and the left leg lead wire is connected to the left leg electrode. The lead wires are designated in various manners; by letters, such as RA for right arm, LA for left arm, and LL for left leg; by using colored terminals, red for the right arm wire, blue for the left arm wire, and white for the left leg wire; or by wires colored in the same manner as the colored terminals.

After recording electrocardiograms of the limb leads, it is necessary to rearrange the lead wires and electrodes for taking the precordial leads. The left leg lead wire is connected to the chest electrode, which is then placed in the proper position on the chest wall. Then the left arm lead wire is connected to the left leg electrode which is still in place. The lead switch is turned to the Lead III position and the chest lead is recorded.

Electrical Interference.—All sources of electrical interference must be eliminated or minimized. Electric fans, electric pads, electric refrigerators, which may be in the room or near by, may cause marked interference with the records, and should be turned off.

The electrocardiograph should be grounded by means of a wire leading from a water pipe, a radiator, or bedspring to the suitable ground wire connecting post in the machine. A water pipe is most desirable for grounding purposes. The electrocardiograph, in the case of the electric type of machine, is connected to an electric light outlet. Be sure to check the type of current available; most instruments require AC current. Some machines are supplied with special bulbs which indicate whether the current is proper for that instrument. If the wrong current is used and the electrocardiograph is switched on, a fuse blows, thus protecting the instrument.

The Room.—The room should be darkened during the actual recording process so as to prevent fogging of the bromide paper by extraneous light. Enough light should be left so that the dials are visible during the operation.

Operating the Electrocardiograph.—The machine is prepared by switching on the electrical current. In the electrical string instrument this starts the timer and throws a beam of light onto the string and the camera. The shadow of the string is seen lying on the zero mark on the scale. This shadow should be focussed so that its outlines appear as sharp as possible.

If the string lies on either side of the zero mark on the scale it must be centered.

Always check the camera box to make sure that the paper is in proper position and that the container which receives the exposed paper is in the open position. If one inadvertently fails to replace the receiving box or if the box has not been opened, the paper may become jammed and stop feeding into the receiving box. This also may occur at times when the paper or film has slipped out of its proper position in the slot.

After the instrument has been connected to the electrodes on the patient and the current has been turned on, it is necessary to ''compensate'' for the skin currents. These currents may be of sufficient magnitude actually to deflect the string shadow completely out of the visual field on the camera, or actually to break the string. This current varies both in direction and in strength in each lead, with different patients, and even with the same lead in the same patient from time to time. The effects of this current can be neutralized by gradually introducing a counteracting current into the system by means of a ''compensating'' dial. In some instruments neutralization of skin currents is accomplished by condensers.

The skin current may cause the string shadow to move to either side of the scale. Many electrocardiographs have the compensator so arranged that by turning the knob clockwise the shadow will move forward, and by rotating it counterclockwise the string shadow moves in the opposite direction. In this way it is easy to determine the direction in which the skin current of the patient deflects the string, and then, by means of the compensator knob, to bring it back to the center.

With the electrodes on the patient connected to the proper lead wires, and the light and timing device turned on, turn the lead selector knob on the control panel to Lead I. Then slowly rotate the ''protector'' knob which shields the string from the current from the patient by means of a large resistance. This gradually reduces the resistance and permits the current from the patient to enter the string. At this time the string shadow will begin to move away from the center line in either direction. During this procedure the string shadow can be brought back and kept on the center line of the camera until finally the protecting resistance is zero and the current from the patient then enters freely into the string. The string shadow then moves in uninhibited response to each contraction of the heart. Now standardize the string by introducing one millivolt current from an independent source of electrical energy such as a Weston cell. If the string jumps more than one centimeter, tighten it by means of a screw or wheel adjustment until the one centimeter deflection is produced. If the string jumps less than one centimeter, loosen it by turning the screw or wheel in the opposite direction until the one centimeter deflection is produced. If the skin resistance is too great, the string must be loosened excessively thus producing overshooting. Under these circumstances the protecting resistance knob must be turned on again and the electrodes reapplied after rubbing more jelly into the skin. It may also be necessary to recleanse the electrodes or lead wire contact points.

With the string standardized, focused, and centered, Lead I can now be recorded. Turn on the camera switch lever. This starts the bromide paper moving at a constant rate, the movements of the string shadow being recorded upon it. The standardization impulse should be recorded while the record of each lead is actually being made.

After photographing the desired number of cycles, usually about ten, turn off the camera lever and again turn on the protector resistance. Rotate the compensator knob to ''zero'' and shift the lead switch to Lead II. Repeat the entire procedure for Lead II, taking care to restandardize and record the standardization for this lead and all subsequent leads. Now take Lead III. It is common practice to have the patient take a deep breath during the recording of Lead III to determine the influence of deep breathing on the electrical axis of the electrocardiogram. With the machine turned off and all the knobs turned to zero readjust the electrodes and the lead wires for recording the chest leads, as indicated above. Now take the chest lead.

Turn the lead selector switch and the compensator switches to zero after the desired leads are recorded. Tighten the string so that it will not be broken by accidental jarring of the instrument. This is done by turning the lead selector switch to zero or to the position marked *4* in some of the electrocardiographs. The string is thus out of the patient's circuit and unprotected by resistance. Tighten it by turning the string tension adjusting wheel until 1 millivolt of current causes only about a 0.3 centimeter deflection. Turn the lever on the record receiver box to the closed position, cutting off the record from the unexposed paper. If more electrocardiograms are to be taken before developing this tracing, turn the lever on the record receiver box to the open position, allowing a new strip of paper to enter the box. Turn off the switch controlling the source of power to the machine and remove the electrodes from the patient. Clean them thoroughly with water, and dry. Remove the electrode jelly from the patient.

With the lever in the closed position, remove the record receiving box and take it to the darkroom for the development of the records.

Developing the Tracings.—Remove the paper from the container in the darkroom and place it in the developing solution. Agitate and examine it from time to time until it shows complete development and proper contrast. With fresh developer and proper temperature of the solution this should take about four minutes. If the temperature of the solution is too low or if the solution is old, more time is required for development. Solutions should be kept at about 68 to 70° F.

Remove the paper from the developer bath, wash it thoroughly in clear water, and then place in the fixing solution. Allow it to remain for about ten or fifteen minutes in this bath. The fixing solution and the developer should be the same temperature.

The white light may now be turned on in the dark room. Remove the electrocardiogram from the fixing bath and wash it thoroughly in running water for about one-half hour. Hang it up to dry. If the developer is too old, the record becomes stained and yellow, and it is necessary to prepare fresh solutions.

Preparing the Tracing for Examination.—Ferrotyping the electrocardiogram will produce a glossy finish and will prevent the records from curling and cracking when they are straightened.

Use a ferrotype plate, 18 by 24 inches, and a squeegee roller. The plate should always be kept clean and glossy. Cut the electrocardiogram into sizes to fit the length of the ferroplate taking care to mark the leads properly so that they will not become confused with each other or with those of other patients. Shake the surplus water from the paper strip and place the strip face downward on the plate. Run the roller firmly over the paper until excess water and air bubbles are forced out and the record lies in perfect contact with the plate. Allow them to dry; the records will of themselves separate from the plate.

The electrocardiogram is now ready to be interpreted by an electrocardiographer. This interpretation is written in special form as directed by the electrocardiographer. Next cut and mount the record, taking care to mark the leads properly and not to mount them upside down.

History.—The date, the patient's name, sex, age, pertinent facts in the history, and type of medication should accompany the electrocardiogram when it is presented for interpretation. Retain the unmounted portions of the tracings, mark them properly, and file them for future reference. Present the mounted record to the referring physician.

THE SANBORN CARDIETTE

Another instrument commonly used is the Sanborn Cardiette. A brief description of this instrument is included as an example of the electron tube amplifier type of electrocardiograph. For complete operating details, consult the brochure which is supplied with the Cardiette.

Be sure that there is enough sensitized paper in the camera and that the batteries are fully charged. Check the camera to see that it is operating. Do not wear a watch while operating the instrument. See that the patient removes his watch.

Prepare the patient and attach the electrodes as directed on pages 2396, 2403, 2405, 2406, 2407 ff.

Turn the voltage knob to the right until the needle on the circular dial, the voltmeter, comes to the position marked 1 MV. The needle must be maintained at exactly 1 MV mark throughout the taking of the tracing. Turning this knob illuminates the left-hand corner of the control panel. If it is not illuminated, start the camera for an instant, then turn it off.

In less than a minute a dark line will slowly move across the scale and out of sight. It soon returns and stops in a visible position on the scale. This is the beam shadow. Turn the beam knob to center this shadow.

Press down on the 1 MV button to cause the beam to deflect. It should "jump" 1 centimeter on the glass scale. If the deflection is over or under the standard centimeter, turn the sensitivity knob clockwise to increase, or counterclockwise to decrease, the deflection, until the 1 centimeter jump is attained. The machine is now standardized.

Attach the lead wires to their respective electrodes, and check them to make sure that they are properly placed.

Turn the lead switch to Lead I. Check the instrument to make sure that the beam is centered and that it jumps the standard 1 centimeter. Turn on the camera, press the lead marker once, and record the first lead, pressing the deflection button once during the recording of this lead to show that the machine is standardized. The average length of a lead is 8 inches, which requires 8 seconds to record.

Turn the camera knob to the "off" position. Turn the lead indicator switch to Lead II. Check the beam for centering and standardization, then turn on the camera and record Lead II. Press the lead marker twice at the beginning of this lead in order to identify it. *Press the beam deflector button once during each lead to show that the instrument is standardized.*

Turn off the camera. Turn the lead indicator switch to Lead III. Center the beam and check the deflection. Instruct the patient to take a deep breath and then to let it out. Turn on the camera and record Lead III. Mark the lead by pressing the lead marker three times. Turn off the camera.

Turn off all switches. Attach the left arm lead wire to the left leg electrode, and the left leg lead wire to the chest electrode. Leave the right arm electrode in place. Put the chest electrode in proper position, turn the lead indicator switch to Lead III, check the beam, turn on the camera, and record Lead IV. Mark by pressing the lead marker button four times. Turn off the camera.

Detach the lead wires and take the instrument or the camera box to the darkroom. Develop the tracing as directed on page 2398.

While making the tracing, if the beam shadow is not in focus, attach the ground wire to eliminate any interfering electric circuits from the outside. If the beam is still not in focus it may be due to skin resistance. Adjust the electrodes so that they are neither too tight nor too loose, or apply more electrode jelly.

Clean the electrode jelly from the patient. Clean and dry the electrodes before returning them to storage.

THE SANBORN INSTOMATIC CARDIETTE

The Sanborn Instomatic Cardiette differs from the Sanborn Cardiette in several details. To operate, turn the main switch to ''STD'' and wait one minute for the amplifier to reach operating stability. Standardize the beam deflection as for the Cardiette. To test the deflection, press down the same knob used to center the beam.

Draw back the autographic slot cover and write the patient's name or identifying number. The camera may be run for a moment if more room is needed for writing. Run a few inches of the paper into the receiving box to prevent ''striking'' the paper with light.

Prepare the patient as directed on page 2403. Plug the patient cable into the rectangular socket at the upper left corner of the panel. Attach the patient cables to the electrodes in the usual manner.

Set the lead length indicator at ''0.'' Each small white mark on the dial represents six inches of exposed paper, or six seconds of recording.

Check the machine for standardization. Be sure that the beam is deflected one centimeter per millivolt. No further adjustment of the sensitivity knob during the test is ordinarily necessary.

The beam deflector button must be pressed once during the taking of each lead to show that the instrument is standardized.

Press and hold down *Instomatic* button at the left of the sensitivity control. With the button depressed, turn the *Lead Selector Switch* to Lead I. Release the Instomatic button. Turn the camera on and record Lead I. Press the lead marker once during taking of this lead. Then turn off the camera.

Repeat for Lead II, pressing and holding down the Instomatic button while turning the main switch to Lead II. Press the lead marker twice for Lead II. Turn the switch to Lead III and repeat for this lead. Press the lead marker three times for Lead III. Turn off the camera between each lead.

To take Lead IV F disconnect the right arm terminal and attach it to the chest electrode. Leave the other connections in place. Apply the chest electrode at the outer border of the apex. Hold down the Instomatic button and turn to Lead IV, then take Lead IV F. Mark by pressing the lead marker four times.

To take Lead IV R, turn the main switch to ''STD.'' Attach LA terminal to the chest electrode. Press and hold down the Instomatic button while turning the main switch to Lead I.

To take Lead IV L turn the main switch to ''STD.'' Attach the LL terminal to the chest electrode. Press and hold down the Instomatic button while turning the main switch to Lead III. Proceed as for other leads.

Run the camera about 4 seconds then turn it off. Turn off all switches. Take the removable record container to the dark room and develop the tracing in the usual manner.

Mounting the Electrocardiogram

Electrocardiograms may be mounted with ordinary photographic mounting tissue. Cut the tissue to desired length and breadth. Attach one corner to the electrocardiogram which has previously been trimmed to desired proportions. Put the tracing on the paper which is to be the permanent record with the mounting tissue beneath the tracing. Cover with a towel or several thicknesses of paper and press with a hot electric iron. Take care not to burn the tracing. Now label each lead. Be sure that leads are not interchanged while mounting them and that they are not mounted upside down.

Maintenance of the Instrument

All electrocardiographs are delivered in proper working condition and are accompanied by a pamphlet giving complete detailed instructions for operating the instrument. Since maintenance and care of the instrument vary with the type of apparatus used, it is best to refer to these pamphlets for such information. In choosing an electrocardiograph it is advisable to bear in mind the availability of service for it.

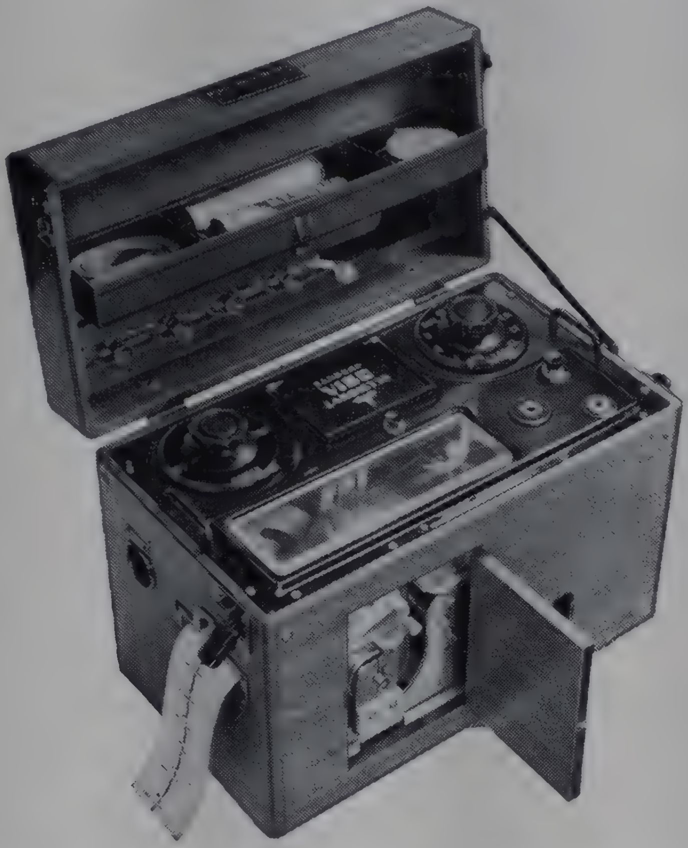

Fig. 729.—Sanborn Model 51 Viso-Cardiette. (Courtesy Sanborn Company, Cambridge, Mass.)

THE SANBORN VISO-CARDIETTE Model 51*

One of the most commonly used types of direct writing instrument is the Sanborn Viso-Cardiette. The Viso-Cardiette produces an electrocardiogram or graphic record of the electrical waves (action potentials) generated by the beating of a patient's heart. The record reads from left to right to represent the passage of time, the standard speed being 25 millimeters per second. The paper is ruled in 1-millimeter squares. The light vertical lines on the graph mark off twenty-fifths of a second, or 0.004 second, per square, and the heavy lines fifths of a second (0.20 second).

The vertical deflections of the recorded tracing show the electrical changes taking place in the heart. Each millimeter square, the lighter lines, represents 1/10 millivolt; each square within the heavier *horizontal* lines represents ½ millivolt. The short lines above the graph printing indicate 3-second intervals.

Complete operating instructions, including checking of the shipment and the instrument, accompany the purchase of such an instrument and will not be repeated here.

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*This section is an abstraction of a pamphlet furnished with the Viso-Cardiette by the Sanborn Company, Cambridge, Mass., and is reproduced with their permission. All cuts courtesy of Sanborn Company.

Operation of the Viso-Cardiette.—

The Viso-Cardiette must not be operated on direct current nor on alternating current having a frequency different from that marked on the serial name plate. While 50-cycle current will not damage a 60-cycle instrument, it will make the recording paper run at a speed equal to 5/6 of the rated value. Direct current will blow the fuse in the instrument.

Remove all watches from the patient and the operator unless they are of the nonmagnetic variety. Watches may be easily demagnetized by gradual withdrawal from the center of an aircore coil carrying alternating current.

Do not expose the instrument unduly to outdoor winter temperatures. It takes some time for the instrument to warm up properly and condensation invites temporary electrical abnormalities.

Technic.—

(1) **Control Tests on Instrument:**

Plug in the power cord and check for proper grounding, as follows:

Make sure the power switch is in the OFF position.

Insert the round plug of the power cable firmly into the power cable socket.

Automatic grounding can occur only if the polarity of the power cord is correct. The grounding indicator is the neon lamp in the grounding disk. Connect the instrument to the line and then touch the gold-plated metal band on the disk with one hand, avoiding contact with any other part of the instrument (so as not to destroy the value of the test; there is no danger of shock), and look at the indicator lamp. If the lamp glows, the polarity is wrong, and the grounding can be corrected by pulling out the power plug and inserting it again the reverse way around. No glow should be obtained now when the metal band on the grinding disk is touched.

Since the grounding can be changed by reversing either end of the power cord, avoid the mistake of marking the power plug at its round end to show which way it should presumably go in.

Turn the power switch to ''ON,'' and observe the writing arm, noting that it wanders here and there, finally becoming stable when the amplifier is fully warmed up. This is normal, and may last for 90 seconds. Prepare the patient while warming up the instrument.

Check the stylus temperature. Locate the stylus temperature control in the covered compartment in the control panel and set the record in motion by turning the power switch to RUN. Lower and raise the stylus temperature by turning this knob to the left and then to the right until the point is reached at which the top and bottom edges of the base line on the record become sharply outlined.

A dull red glow of that part of the stylus ribbon projecting beyond the paper while the record is being run is a good approximate indicator of the recommended stylus temperature.

Check the lead marker, while the record is running, by depressing the lead marking button in the center of the control panel once or twice. Small black marks will appear at the top of the record, above the printed scale. Stop the record by returning the power switch to the ''ON'' position. The amplifier will remain in operation until the switch is turned to ''OFF.'' An automatic switch reduces the stylus temperature while the record is not running, preventing the stylus from burning a hole in the paper.

Adjust the position of the writing arm approximately to center by the centering control in the small compartment.

If the sensitivity has been adjusted when the instrument is first set up, it will seldom need correction. The universal standard is 1 centimeter per millivolt. While the 1MV button at the right end of the control panel is being depressed and released rapidly, observe the writing arm. Adjust the sensitivity control until the deflection sweeps through 10 of the small squares (2 large squares) on the recording paper.

By turning the control counterclockwise, the deflection is decreased; turning it clockwise increases the deflection.

Check the final standardization performance while the record is running. Measure deflections from one edge of the base line to the same edge of the deflection. If the vertical portions of the standardizing deflections are not visible, increase the stylus temperature until they are.

All routine tests must show standardization, so that 1 centimeter of deflection represents 1 millivolt of electrical potential at the patient's electrodes.

Try to apply the millivolt pulse standardization immediately after the T wave.

(2) **Preparation of the Patient.—**

The patient should be warm, comfortable, and relaxed, not apprehensive.

All three standard limb leads must be connected for all patients, even if only a chest lead or a single limb lead is to be recorded.

It is important to have good contact between the skin and the electrodes. Use the Redux electrode paste, or any suitable electrocardiographic jelly. Apply it sparingly and only where needed. Remove bracelets and wrist watches and exposed clothing as a general precaution.

Expose both forearms and the calf of the left leg. If chest leads are required, arrange the clothing so that an electrode can be applied to the chest.

Spread a moderate amount of electrocardiographic jelly on the skin on the inner side of one forearm, about half way between wrist and elbow.

Holding the electrode so that one long edge can make contact with the patient's skin, scrape the edge upward gently but firmly, along the patient's arm, for a distance of about 2 inches.

With the opposite edge of the electrode, scrape downward on the patient's arm. Using about 10 or 12 strokes, continue with a stropping motion until the jelly is well distributed, and the skin slightly reddened. This improves electrical conductivity.

Make sure there is a slight, moist residue of jelly remaining.

Fasten the electrode in place with one of the rubber straps, the concave surface being lengthwise on the arm and the thumbscrew nearest the patient's hand.

Hook the strap over the central pin on the electrode.

Draw the strap tightly enough to produce firm contact between the electrode and the skin, but not so tightly that it is uncomfortable.

No metallic particles should be trapped under the electrode and the skin should be clean. If necessary, swab down the electrode sites with 70% alcohol.

Place a second rectangular electrode on the other arm, using the same method. Place a third on the left leg, with the thumbscrew pointing upward.

If AV leads or V (''Wilson'') chest leads are to be taken, apply an electrode also to the right leg. This is essential to eliminate automatically AC interference when AV and V leads are being taken.

Place the leg electrodes to one side of the calf. More friction must be used on the legs than on the arms. Do not remove hair; simply rub thoroughly.

The small disk electrode is provided for chest leads. The edge of this may be used for scrubbing the skin, as with the limb leads. No surplus paste should extend beyond the disk, for the chest leads are essentially *point contacts,* and an excess of paste is the equivalent of a larger electrode and may distort the result.

It is best to hold the chest leads in place by means of the chest strap and not with a folded towel.

When the electrodes have been placed, plug the patient cable into its receptacle and connect the free ends of the leads to the appropriate electrodes as indicated by the markings: RA to right arm, LL to left leg, and so on. Screw the nut of each terminal down firmly onto the tip of its lead wire.

If the RL lead is not required, the wire may be unscrewed from the distributor block and stored.

(3) **Recording the Electrocardiogram.—**

After the lead wires have been connected and the patient is comfortable and relaxed, the instrument is ready for operation.

Turn the power switch to RUN for a moment, until a short length of record protrudes from the case. Check the stylus temperature and the standardization during this time, by depressing the "1 mv" button a few times and inspecting the deflections.

Return the power switch to "ON."

Write the date and the patient's name on the protruding strip of record. Record any other data desired.

With one hand, push the Instomatic switch (just below the lead selector switch) and with the other turn the lead selector switch to the first desired lead position, usually lead 1. Keep the Instomatic switch pushed for a second longer, then release it.

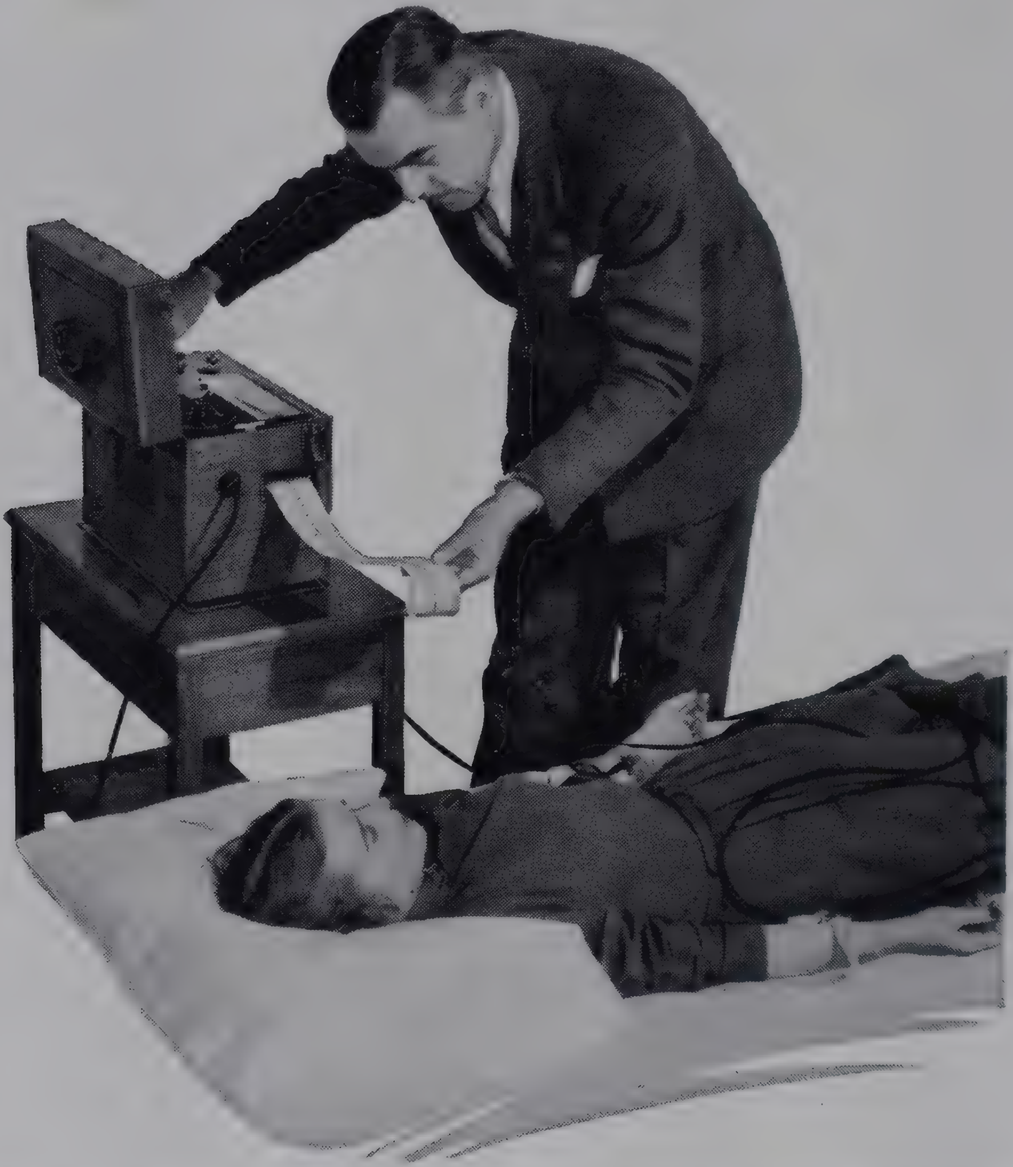

Fig. 730.—Sanborn Model 51 Viso-Cardiette in use. (Courtesy Sanborn Company, Cambridge, Mass.)

Observe the deflections of the writing arm through the observing window, and if they are of unusual amplitude, adjust the centering control so that the extreme deflections in the upward and downward directions are about equally distant from the margins of the graph. If the deflections are well within the ruled area of the paper, exact centering is unimportant.

If the stylus drifts or thrashes from side to side, or shows signs of tremor or of electrical interference, turn the selector switch back to "STD" and proceed as directed below under "remedies."

When the apparatus is in order, start the record by turning the power switch to "RUN."

Mark the lead being used by briefly pressing the lead-marking button at the center of the instrument panel, once for Lead I, twice for Lead II, etc.

Register the sensitivity as directed above. Depress the "1 mv" button briefly between two heartbeats. Repeat this in each lead.

Do not disturb the sensitivity control.

Permit the record to run about 8 to 10 seconds, giving 8 to 10 inches of record.

Stop the record by turning the power switch from "RUN" to "ON."

Proceed to Lead II.

Push and hold the Instomatic switch and turn lead switch to "2." In a second, release the switch.

Observe the writing arm movement.

Turn the power switch to "RUN." Mark the lead. Register the sensitivity. Run about 8 to 10 inches of record.

Repeat in the same manner for Lead III.

Continue for the AV lead if so directed. No change of electrodes or electrode connections is required to secure proper registration of the AV leads, except that a connection MUST be made to the patient's right leg, to properly ground the instrument.

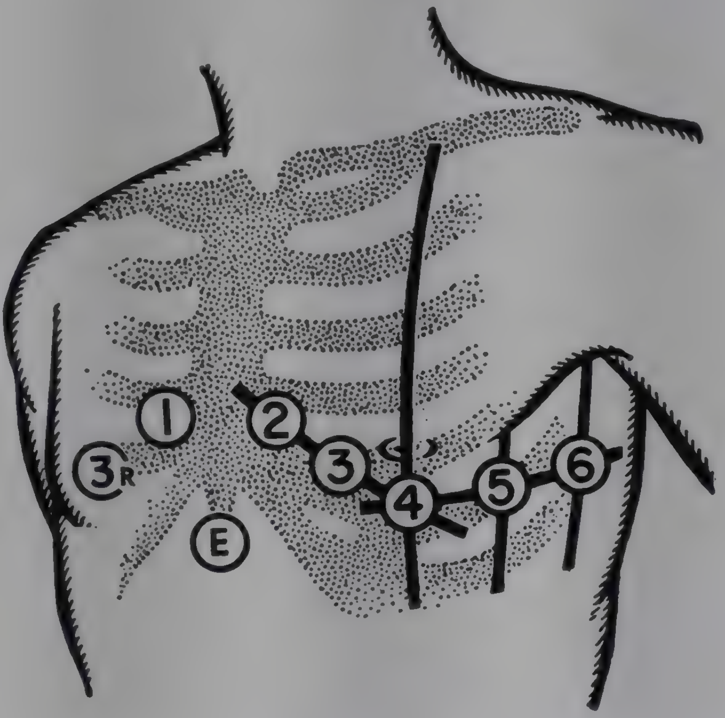

Fig. 731.—Position of electrodes for the chest leads. (Courtesy Sanborn Company, Cambridge, Mass.)

(4) **Chest Leads.**—(See pages 2407 ff. for Unipolar Leads.)

The physician determines and directs which chest leads are to be recorded, and in what sequence. He also determines how many electrode locations are to be recorded. Placing of these locations are shown in Fig. 731.

The Viso lead selector switch is provided with positions for taking both the conventional chest leads (CR, CF, and CL) and the Wilson or Vector return chest leads (V).

The "C" or chest connection must be attached to the chest electrode and placed and held on the chest wall. In addition the RL must be connected to the patient's right leg whenever V chest leads are to be taken, for the purpose of grounding the apparatus.

Before recording any chest lead, carefully observe deflections of the writing arm, and if necessary use the centering control to shift the base line to a position which will keep the deflection peaks approximately equidistant from the edges of the recording paper.

On positions CR, CF, and CL, the switch automatically connects the patient to the instrument for recording conventional chest leads. If the chest electrode has been placed at the apex, position CF will record chest (apex)-left arm, conventionally designated CF_4; CR would represent chest-right arm; and CL, chest-left arm.

The designation "V" is used with reference to the unipolar leads, which record the potential variations at any single point on the body, whether on a limb or at a chest-electrode site. An inactive or neutral reference point is obtained by connecting the three limbs RA, LA, and LL, each through a separate high resistance, to a common junction, which can then be regarded, without significant error, as a reference point of zero potential. This is the circuit suggested by Wilson and associates.[1] The lead selector switch automatically incorporates the ''Wilson circuit'' when turned to the V position.

Connect the exploring unipolar electrode to lead wire ''C'' of the patient cable, and then place at one or more of the chest locations. See Fig. 731. The recordings obtained represent the potentials present at the selected numbered sites, and are customarily referred to as V_1, V_2, etc.

(5) **Special Leads.—**

For esophageal or other special leads, the chest electrode connection is used for the ''exploring'' electrode, with the lead selector switch on ''V'' or on some other position, according to the technic being employed.

To mark these special leads, write directly on the record, designating the lead being taken. This may be done while each lead is being recorded, or on completion of the entire record strip.

In cases of amputations, use the remaining stump if it is long enough to allow the rubber electrode strap to take hold. The right leg may legitimately be subsituted for the left, although the two arms are not interchangeable. In difficult cases, fasten an electrode with adhesive tape on the shoulder or hip where the limb was formerly attached. If the Wilson circuit is to be used with one leg absent, put both the ''RL'' and ''LL'' electrodes on the remaining leg, but at sites separated by six inches or more, and with no electrode paste on the skin between them.

(6) **Recording Chest Leads.—**

To record the various chest leads, after the electrodes have been attached, push the Instomatic switch, and turn the lead selector switch to the desired position.

Record the lead as outlined for limb leads.

While the chest electrode is being shifted, to avoid disturbing the Viso-Cardiette amplifier and thus keep it ready for immediate recording of each chest electrode location, the Instomatic switch is provided with a protective locking feature. Pull the switch toward the operator to the locking position, before moving the electrode, each time a new chest location is to be recorded.

(7) **Completing the Record.—**

Tear down on the record when complete. Return the lead selector switch to STD and turn the power switch to OFF.

Disconnect the patient and sponge off the electrode sites with a damp towel.

Wash the electrodes so the paste residues will not accumulate on them. DO NOT USE STEEL WOOL.

(8) **Cleaning Up.—**

Disconnect the patient from the apparatus and sponge off the electrode sites with a damp towel. Wash the paste residues off the electrodes, but DO NOT USE STEEL WOOL. Dry and store.

(9) **Maintenance of the Instrument.—**

When the Viso-Cardiette is not in use, keep the cover closed, and further protect it with the gabardine slip cover. The **electrodes** must be kept clean and ''shiny-bright.'' Wash thoroughly after each use in hot water, and make sure all the Redux paste has been removed. Dry and replace in the instrument. If necessary, scrub with a scrubbing brush, *NOT* steel wool, using strong soap. Polish with a little Redux paste, wash *thoroughly*, and dry. Clean sandpaper may also be used.

Keep the **patient cable tips** clean in the same manner as the electrodes. Clean periodically with a strip of sandpaper but *NEVER* with steel wool.

No oiling of the instrument is ever required.

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[1] Am. Heart J. 9: 447, 1933.

To clean the **stylus ribbon**, connect the instrument to power, turn the power switch to *ON* and the lead selector switch to *STD*. Open the paper supply door, lift the stylus off the paper by turning the galvanometer tilting lever counterclockwise, turn the heat control to maximum, and the power switch to *RUN*. Let the paper run through for 10 seconds, holding the stylus off the paper. The excess heat will melt off the plastic accumulation on the ribbon, and a clear, sharp base line will result.

If the base line is poorly defined, this might be due to several factors. (1) Insufficient heat. If the stylus temperature has inadvertently been moved, determine this by running some paper through, while turning the control left and right until the desired blackness is secured. (2) Clean the stylus as directed above. (3) Insufficient pressure. Remove the chassis from the cabinet, turn the knurled thumbscrew, which controls the pressure of the stylus, clockwise until the stylus ribbon clears the paper, then turn it counterclockwise until the ribbon just meets the paper. Place a pencil mark on the thumbscrew to note position and then turn the screw 1¼ additional turns, counterclockwise. (4) If the ribbon is slightly bent, the writing arm must be removed. After removal, place the arm, held near the base, on a flat surface, and *gently* smooth its upper surface to make it evenly flat. Then replace the arm.

To change the recording paper, open the galvanometer viewing window, open the paper drive door in front of the cabinet, swing the galvanometer tilt handle a half turn counterclockwise, and remove spool support bracket by pulling up and out. Raise the tension plate by lifting on the thumb latch until the black roller guide plate can be seen and heard to "snap up." Remove the remaining paper, while still lifting on the thumblatch, by rotating the spool counterclockwise and sliding out the spool with shaft and core. Grasp the core and shaft in one hand, and with the other turn the spool clockwise, and at the same time pull out.

To install new paper, remove the wrapping from the roll of Permapaper. Grasp the roll with one hand and the spool with the other. Push the shaft into the core of the paper roll, at the same time turning the spool clockwise. Continue until the paper is flush against the spool. Lift the tension plate slightly and slide the shaft, with the roll of paper, onto its spindle, pushing in as far as the roll will go. Then turn the spool clockwise so that about an inch or two of paper (no more) with the *unprinted* side *up* projects beyond the tension plate, toward the galvanometer magnets. Using the left hand, rotate the roll of paper on the spindle clockwise while, at the same time, the right hand holds the end of the paper, and guide the paper up and over the horizontal writing paten. Hold the right hand on the paten, and, with the left hand, place the edge of the paper underneath the roller guide plate, working from the top through the open viewing window. Obtain additional slack by rotating the roll of paper clockwise, or by pulling the edge or end gently. If too much slack is obtained, rotate the roll counterclockwise. Holding the roll in position, inspect the paper throughout its exposed length to make sure it is smooth and even. Still holding the paper in position, with the other hand press the roller guide bar down firmly until it snaps into position. Reinstall the proper spool support bracket by placing the slot over the rod in the base, at the same time raising the tension plate slightly. Turn the power switch to ON. While the paper is moving, press and hold the forefinger against the roll of paper. Any slack will now be taken up automatically. If necessary, use the fingers to guide the paper through and out the discharge slot. If the paper does not feed through automatically repeat the process from the beginning. Finally rotate the galvanometer tilting handle counterclockwise one-half turn, and close the viewing window.

UNIPOLAR LEAD ELECTROCARDIOGRAPHY

In the last ten years, there has been a steadily increasing interest in and awareness of the important unipolar lead electrocardiography so that at present this method has been universally adopted. These leads have been shown to be advantageous for a number of reasons. Among the most important is the light that they have thrown on the significance of abnormalities of the Q waves, particularly in Lead III in the diagnosis of posterior wall infarction, the differentiation of pulmonary artery obstruction from posterior myocardial infarction, the diagnosis of bundle branch block, right ventricular hypertrophy, and the detection of abnormalities when the standard lead electrocardiogram is normal or shows questionable changes. The effect of changes in position of the heart are particularly demonstrable with these leads.

Method of Taking Unipolar Leads.*—

A unipolar lead is one which represents the heart voltages present at *one* particular spot on the body with respect to a nonfluctuating reference point. This reference point is secured by connecting the limb electrodes together through three equal resistances of 5,000 ohms or more; the junction is the reference point, or central terminal, which is usually embodied in the lead selector of the electrocardiograph, or in a special switching box.

The electrodes placed on the body are then paired, in turn, with this reference point or central terminal, to secure the unipolar leads which represent the actual voltages present at these single points on the body.

With limb leads, however, these voltages may be rather small, so that special steps must be taken to secure a record with complexes of sufficient height or amplitude for easy and accurate measurement.

Leads V_R, V_L, V_F:

Leads V_R, V_L, and V_F are unipolar limb leads, in which the pairing is achieved by placing an additional ''exploring'' electrode (normally attached to the ''C'' wire of the patient cable) on the limb being measured, slightly removed from the electrode already in place. Do not allow electrode jelly to communicate between electrodes.

With the lead selector switch placed on V, a unipolar limb lead will be secured. Special steps must be taken to produce a record with complexes of ''readable'' amplitude. Therefore, in taking these leads, it is customary to increase the sensitivity of the electrocardiograph by 50%, or from 1 cm./mv. to 1.5 cm./mv. Allowance must be made for this increase, since the amplitude of the waves will show a voltage of 1.5 times that which is actually present at the location being recorded.

''R'' designates the right arm, ''L'' the left arm, and ''F'' the left leg (or foot).

Leads aV_R, aV_L, aV_F:

Leads aV_R, aV_L, and aV_F are also unipolar limb leads, and now are the ones most widely used. They are referred to as ''augmented'' unipolar limb leads.

No special placement of electrodes is required, nor is it necessary to change the sensitivity of the electrocardiograph to secure the increased or ''augmented'' height of the complexes. All necessary functions are performed automatically within the instrument, or within a special lead selector box, interposed between the patient and the electrocardiograph.

Turn the switch to the desired position—aV_R, aV_L, aV_F—to obtain the augmented unipolar limb leads.

The terminal letters designate the extremity, and are paired with the central terminal or reference point.

Allowance must be made in measurement and interpretation for the 1.5 cm./mv. increased sensitivity.

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*With permission of Sanborn Company, Cambridge 39, Mass. From a bulletin entitled "Nomenclature and Derivation of All Presently-Used Leads" (1953).

Leads V_1, V_2, etc.:

Leads V_1, V_2, etc., are unipolar chest leads. They are achieved by pairing a point on the chest wall (see Fig. 731) with a neutral of zero reference point.

The subordinate numerals—V_1, V_2—are used to designate the position of the chest or exploring electrode.

Leads V_{3R}, V_{4R}, etc.:

Leads V_{3R} and V_{4R} are unipolar chest leads, as described above, but taken from positions on the chest wall to the right (R) of the sternum.

High V Leads:

High V leads are unipolar chest leads, taken with the chest or exploring electrode at positions on the chest wall one interspace above positions V_{1-6} and designated as high V_3, high V_4, etc.

V_E or V Ensiform Lead:

V_E or V ensiform lead is a unipolar chest lead, taken with the exploring electrode placed on the chest wall at the ensiform (base of sternum) position.

Because of the method by which unipolar leads are obtained, their patterns can be explained in terms of easily understandable principles.

Since the indifferent electrode (of Wilson) is for practical purposes zero (though not absolutely so), the actual values of the potentials at each extremity can be determined with unipolar leads. This is in contrast to the conventional bipolar leads (the Leads I, II, III, and the conventional chest leads), in which the potential recorded is the *difference* in the potential generated at each of the two electrodes in the bipolar leads.

In general, the relationships of the standard leads and the unipolar extremity leads are such that one type can be derived from the other as follows. The pattern of Lead I would be the potential values at the left arm (V_L) minus those at the right arm (V_R). The values of the potentials in Lead II would be the potentials recorded at the left leg (V_F) minus those at the right arm (V_R). The potentials recorded from Lead III would be the values derived from the left leg (V_F) minus those from the left arm (V_L). Thus it can be seen that the three standard bipolar leads consist of the algebraic summation of the unipolar leads.

Unipolar limb leads localize the electrical activity of the various areas of heart muscle. Thus, a unipolar lead from the left arm (V_L) records the potentials generated in the left lateral wall of the heart; a unipolar lead from the left leg (V_F) records the potentials produced at the diaphragmatic surface; the unipolar lead from the right arm reflects the potential variations of the atrial and ventricular cavities. Change in position of the heart with consequent altered relationships of the positions of these various regions of the heart with respect to the unipolar electrodes would alter the configurations of the electrocardiographic patterns in certain well-defined ways according to whether the heart is vertical, horizontal, or rotated either clockwise, counterclockwise, or backward or forward. It is beyond the scope of this chapter to describe and illustrate the different patterns of the unipolar leads according to the various positions of the heart and the modifications of these patterns produced by disease processes. This phase of electrocardiography has been thoroughly described in many books devoted to this subject.

INTERPRETATION OF THE ELECTROCARDIOGRAM

THE NORMAL ELECTROCARDIOGRAM[1]

The electrical activity in the heart muscle which precedes each contraction of the heart produces deviations in the electrocardiogram which are in general similar for all normal electrocardiograms. Nevertheless the electrocardiogram of every individual exhibits minor variations which are almost as characteristic for that individual as are his fingerprints. This is true only in so far as no alteration in the physiologic or anatomic state of the heart has occurred. The many variations of the normal electrocardiogram must be studied in order that proper interpretation of tracings may be made. Not infrequently erroneous conclusions, with all their serious implications, have been drawn by inexperienced persons who attributed minor deviations within the normal limits to organic heart diseases. Mistakes have also been made in the improper differentiation of abnormal tracings due to altered physiologic states from those due to actual organic disease.

Waves.—The series of waves seen in electrocardiograms of normal heart cycles was arbitrarily named by Einthoven as the P, Q, R, S, T, and U waves. This is the conventional terminology which has been universally adopted.

The form, amplitude, and direction of the different waves vary in the different leads. The amplitude of the deflections in Lead II is equal to the sum of the amplitudes in Leads I and III. This knowledge is helpful in correcting errors which are sometimes made in labeling and mounting electrocardiograms of the conventional three leads.

The P wave is produced by electrical activity spreading through the auricles. It is a small, rounded deflection which varies in height from 0.5 to 3.0 millimeters, representing 0.05 to 0.3 millivolts. Duration or width of this deflection normally does not exceed 0.1 second. At the end of this deflection when the string shadow has returned to the baseline or isoelectric level, there is a period in which there is no deflection whatsoever from the isoelectric level until a group of sharper, taller waves, the QRS complex, appears.

The QRS deflections are due to the onset and spreading of electrical activity throughout the ventricles. The interval between the onset of the P wave and the onset of the QRS complex is called the P-R or P-Q interval. The Q wave is often absent normally; under these circumstances the P-R interval is measured from the onset of the P wave to the onset of the R wave. If the Q wave is present, this interval is called the P-Q interval and is measured to the onset of the Q wave.

During this P-R interval the impulse is traveling through the auricles to the ventricles along a set pathway known as the bundle of His and the A-V node. The P-R interval measures this time, which is normally from 0.12 to 0.20 second. The QRS complex varies normally in certain respects—whether the initial deflection is directed upward or downward, whether the terminal deflection is downward (invert), or whether there are several upward or downward deflections. By convention the first deflection, if upright, is known as the R wave; if downward, the initial deflection is known as the Q wave.

The downward deflection which follows the R wave is known as the S wave. The S wave may often be absent. Not infrequently a second upward deflection occurs following the S wave. It is known as the R prime (R′) wave. If there is still another downward deflection following the R wave it is called the S prime (S′) wave.

Since the QRS complex represents the *spread* of the action current throughout the ventricular musculature from start to finish, the time interval from the onset of QRS, whether it begins with a Q or with an R, to the end of its final deflection, either with an R or with an S, measures the speed of this electrical impulse through the ventricles. Normally this time interval does not exceed 0.10 second.

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[1] Assemblage of tracings in this chapter by Margaret Peteler, Technician in Electrocardiographic Laboratory of Jewish Hospital of St. Louis.

The R wave normally varies in height from 5 to 15 millimeters. After the electrical disturbance represented by the QRS has subsided, there is a segment, known as the S-T segment, which is usually isoelectric and of rather variable length; it ends in a rounded wider deflection known as the T wave. The ventricles are depolarized for the duration of the S-T segment and have not begun to recover their state of electrical excitement. This duration varies greatly depending upon heart rate, age, and sex.

The interval from the beginning of the QRS complex to the end of the T wave is known as the Q-T interval. It represents the duration of the spread of the electrical excitation and then the recovery from this state of the ventricular musculature. This varies with heart rate, age, and sex, but for normal rates it is usually about 0.4 second. The T wave, which is the final evidence of electrical imbalance in the ventricles, represents the phase of recovery from electrical excitation in the ventricles. During the period of the T wave the musculature approaches and reaches the completely polarized state. At the point of complete polarization the T wave has receded to the isoelectric level, and there are no deflections because the electrical potential is equal throughout the ventricles. The T wave in normal electrocardiograms is upright in Leads I and II, but in Lead III (called the "lead of exceptions" because of its variability under normal conditions) it may be upright or inverted. The amplitude of the T wave for the conventional leads varies from 1 to 6 millimeters.

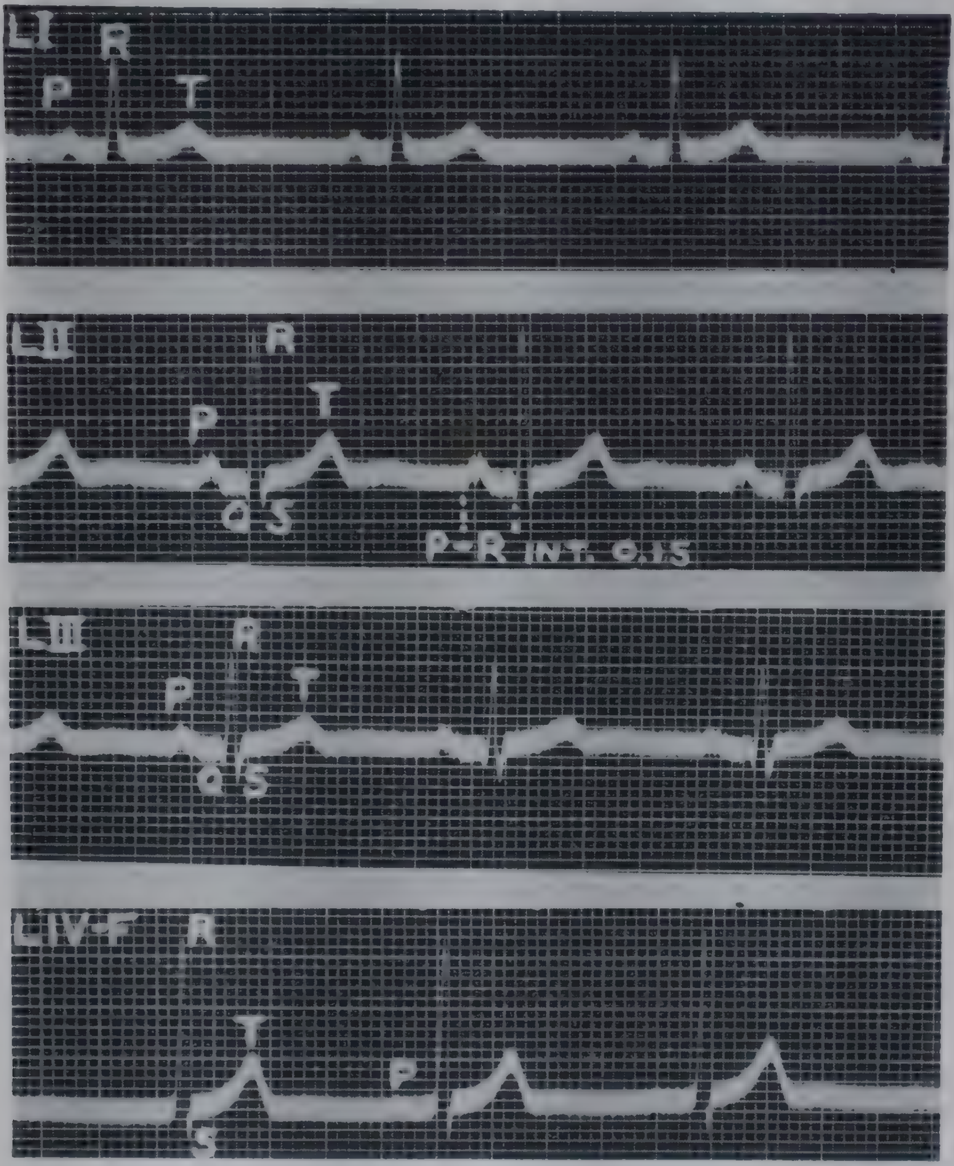


Fig. 732.—Normal electrocardiogram. P, Q, R, S, and T waves appear in proper relationship and are of normal configuration and amplitude. Note that the amplitude of T in Lead II approximates the sum of the amplitudes of T in Lead I and Lead III. Note the practically isoelectric P in Lead IV F which is a normal occurrence. Absence of Q in Lead IV F is normal. Presence of a QIV of 2 or more mm. is abnormal.

A low upward wave often shortly follows the T wave. This is known as the U wave. Thus far the cause of this deflection has not been definitely ascertained. The U wave occasionally is inverted. When large or asymmetrical, it is sometimes associated with myocardial damage.

The chest or standard bipolar and most recently unipolar precordial leads have come into common clinical usage since 1932. They have been invaluable in the diagnosis of myocardial infarction. Two electrodes are used to take bipolar precordial leads. One, the exploring or chest electrode, is placed in various positions to be described. The other, the indifferent electrode, is placed over a point distant from the heart.

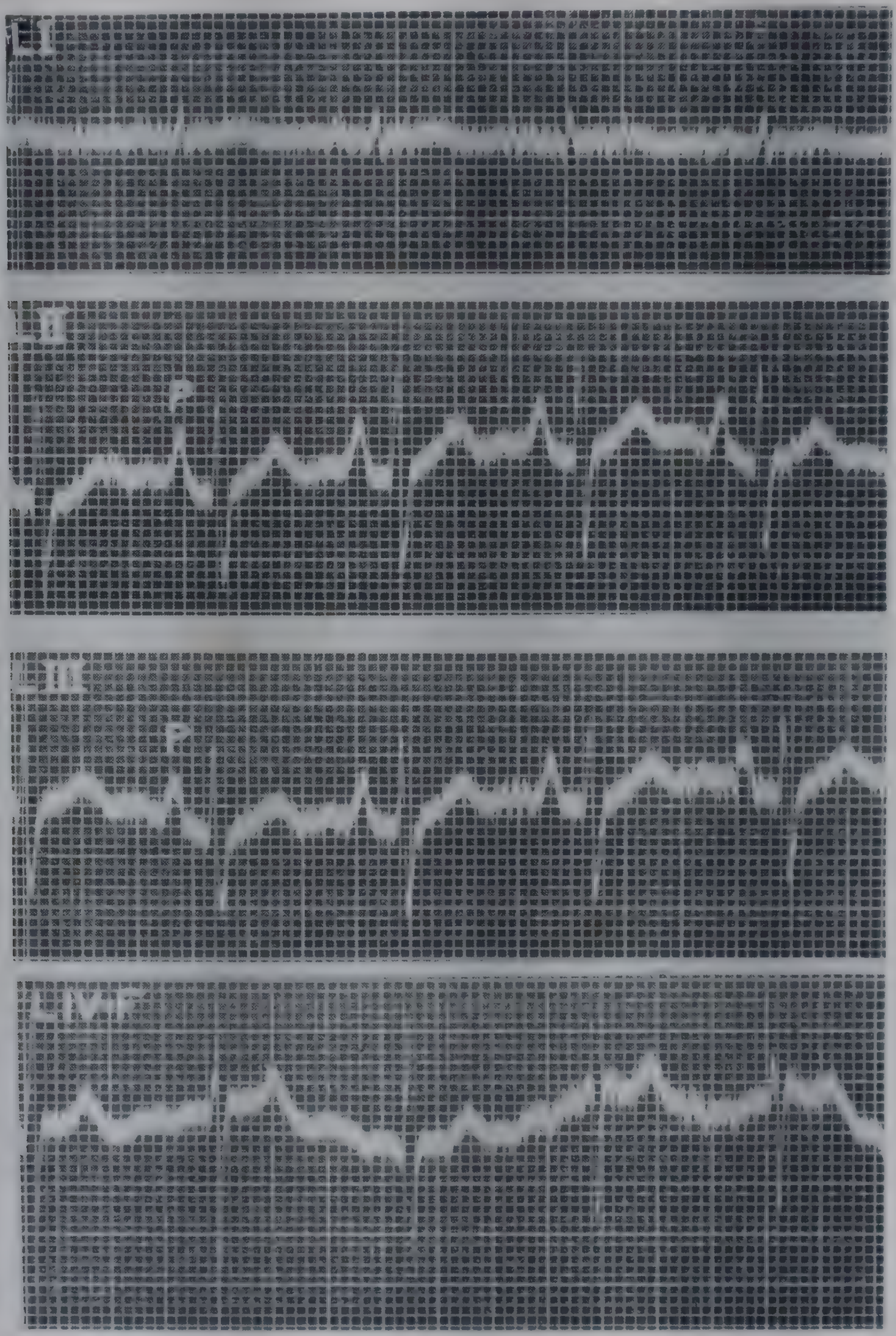


Fig. 733.—Artefacts due to muscular tremors. In addition to the artefact characterized by fuzziness of the tracing due to muscular tremor, note the prominent sharply peaked P waves in Leads II and III characteristic of the so-called P-pulmonale.

There are various types of bipolar chest leads each designated according to the position of the chest (exploring) electrode and the indifferent electrode. These various positions of the electrodes are designated as follows: The Roman numeral IV is used to indicate a chest lead, and a letter follows the numeral IV to indicate the position of the indifferent electrode. If the indifferent electrode is on the left leg, the lead is called IV F; if on the right arm, it is IV R; if on the left arm, IV L; and if on the back, IV B. Another type of lead which is most accurate is that advocated by Frank Wilson and his co-workers. It is a central terminal lead or V lead. In this lead the indifferent electrode is formed by connecting the right arm, left arm, and left leg electrodes together. This type of connection is advantageous in that the potential of this indifferent electrode is more nearly zero than when the indifferent electrodes are placed otherwise, where the current from the heart exerts some influence on the distant electrode though not nearly so much as on the exploring or chest electrode.

The chest lead may be recorded with the exploring electrode placed over different areas of the chest in relation to the heart. These positions on the chest are designated by numerals following the letters indicating the type of chest lead. They include the first letter C to indicate a chest lead, followed by the letters F, R, L, B, or V to indicate the position of the indifferent electrode as described above.

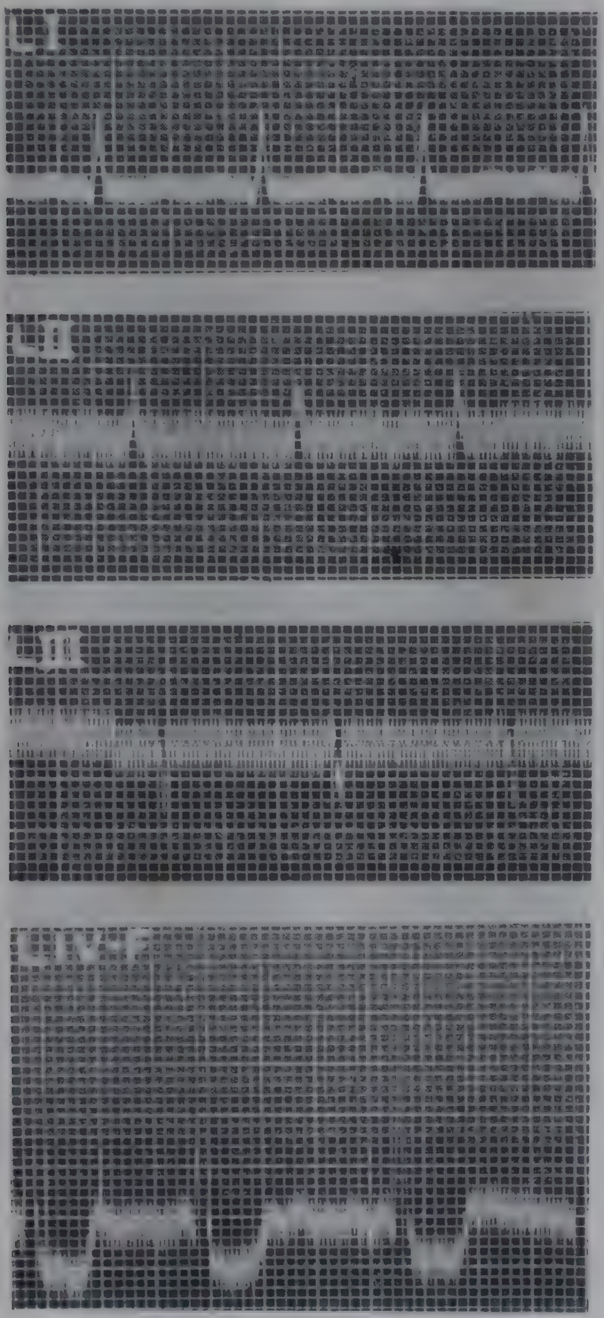


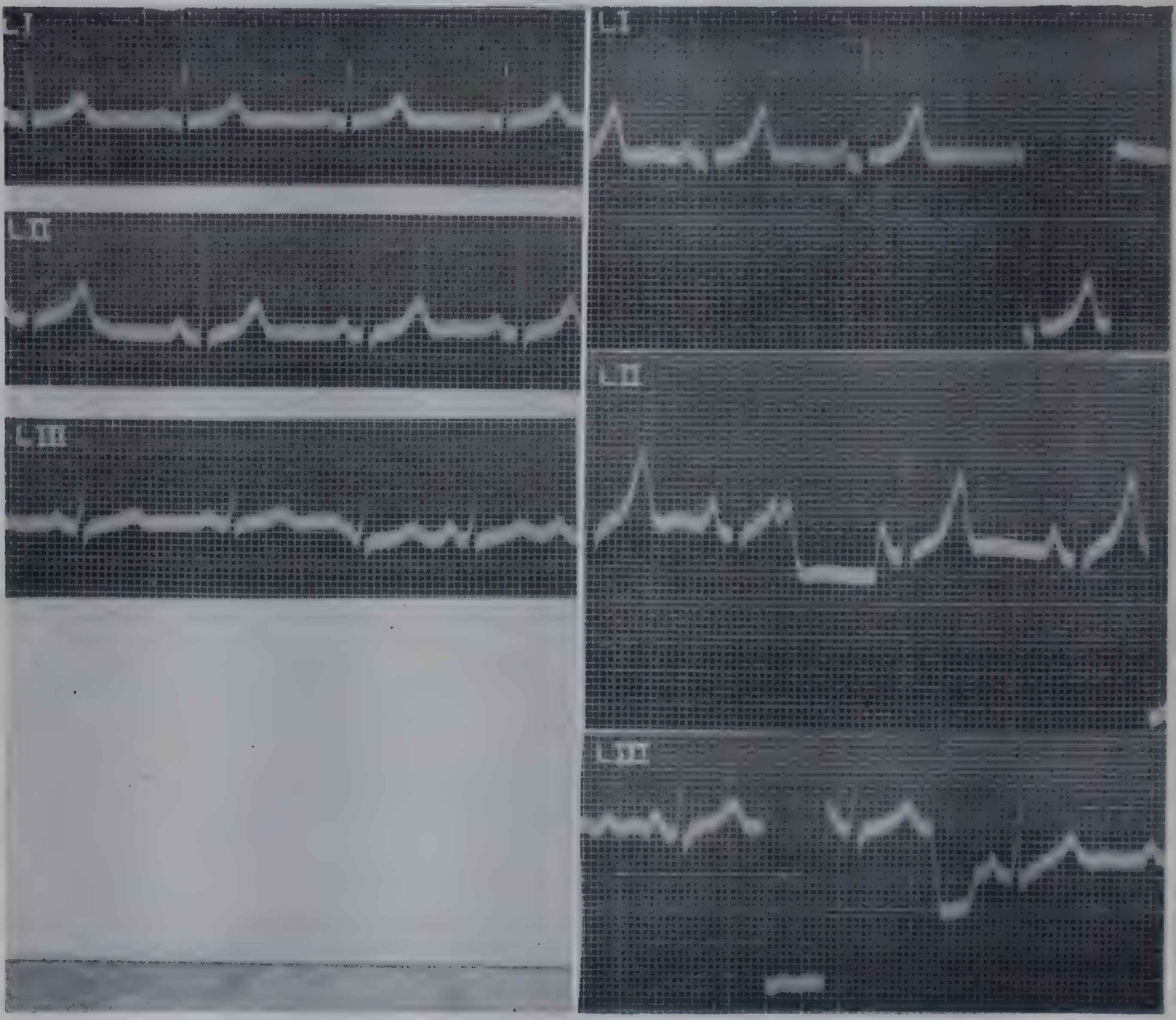
Fig. 734.—Artefacts due to interfering alternating current. This is evident chiefly in Leads II, III, and IV F.

If the chest electrode is placed in the fourth intercostal space just to the right of the sternum, the numeral I then follows. If the position is the fourth interspace just to the left of the sternum, the numeral II is used. When the position of the chest electrode is midway between that of numeral II and the midclavicular line in the fifth interspace, it is designated as III. Position IV is the fifth interspace, midclavicular line; position V is at the same horizontal level in the left anterior axillary line; position VI, the same horizontal level as V, left midaxillary line.

If, for example, the indifferent electrode is placed on the left leg, the various chest lead positions are recorded as CF_I, CF_{II}, CF_{III}, CF_{IV}, CF_V, and CF_{VI}, respectively.

To clarify these designations, let us emphasize the distinction between the leads CF_{IV} and IV F. With the first designation, the chest electrode is always in the fifth interspace, left midclavicular line; with the latter the chest electrode is placed over the heart apex wherever it may happen to be.

Various positions on the chest are utilized because it has been found that some lesions of the myocardium are more apt to be reflected when the chest lead is placed in one position than when placed in another. Positions for the chest lead other than those previously described may sometimes be advisable when attempting to localize the site of the lesion or to determine the type of bundle branch block, if present.



A. *B.*

Fig. 735.—Artefacts due to a loose string. In *A* the electrocardiogram has been properly standardized. In *B* the string has been overstandardized, the string being much too loose. Note that the P, QRS, and T waves are altered in size and form.

As stated above, esophageal leads are sometimes desirable. In these instances a specially designed exploring electrode is swallowed until it reaches the level of the heart. The P waves are prominent in esophageal leads; for this reason this lead may be decisive in determining the nature of an arrhythmia in which P waves cannot be definitely identified in the conventional leads. This lead may also be of value in identifying posterior myocardial infarction, since the esophagus is more nearly in apposition to the posterior ventricular wall.

The appearance of the normal tracing taken with the chest leads varies in many details with the position of the electrodes. It is important, therefore, that the electrocardiographer familiarize himself with these normal variations. Since these variations are discussed in full in books devoted to electrocardiography they are not presented in this chapter. The characteristics of Lead IV F are briefly described because Lead IV F is the one that has been used in taking the electrocardiograms reproduced in this chapter. In the last several years the unipolar chest leads (V leads) have generally replaced Lead IV F in common usage. What is said in describing the characteristics of the IV F largely applies to the analogous unipolar chest leads (V_4).

Characteristics of Waves in Lead IV F.—The P wave is usually quite small and may be inverted, diphasic, or upright. In most instances it is inverted.

The P-R interval is as described for the conventional leads.

The QRS complex is usually of greater amplitude and tends to be slightly greater in duration than in the conventional leads. If R is less than 1 mm. in height, it is considered definitely pathologic. The S may be greater in amplitude than R, normally.

Q is usually absent but when present is not greater than 2 mm. in depth. A Q wave deeper than this is usually significant of myocardial damage as a result of coronary artery involvement. If a Q wave is present in Lead I, there tends to be a Q in Lead IV F.

The S-T segment may be short or absent, and normally is often elevated to a slight degree.

The T wave is characteristically larger than in the conventional leads, usually 5 to 7 mm., and is almost always upright in the adult. In young children the T wave is not infrequently inverted. Although it has been stated that the T wave is rarely notched or diphasic it has been Elson's experience on occasions to find notched and even slightly diphasic and inverted T waves in the absence of organic heart disease.

The U wave is often quite prominent in Lead IV and at times its appearance shortly after the T wave gives a simulation of a terminal diphasic tendency to the T wave.

In CF I, T is normally often inverted, R is usually of low amplitude, and S is relatively deep. In CF_4, which is taken with the electrode in the midclavicular line, fifth interspace, the appearance is the same as in IV F with the electrode over the apex, provided the apex is in its usual position in the midclavicular line, fifth interspace. Leads CF V and CF VI give an appearance similar to that of Lead I except that the amplitudes of R, S, and T tend to be smaller. The rule is that with the exploring electrode to the right of the sternum the tendency is for R to be small, S to be deep, and T to be inverted; and as the electrode is moved to the left axillary line the R becomes relatively tall, the S relatively small, and the T upright.

Common Technical Difficulties.—In order that a proper electrocardiogram be recorded it is necessary, when making the tracing, to understand some of the common technical difficulties which may cause artefacts or external deformities of the record, making proper interpretation difficult or impossible.

1. **Deformities due to muscular movement of the patient.** The movement of an arm or leg may cause slipping of the electrode over the skin, with sudden marked or slight shifting of the string from the base line. Slighter muscular contractions such as muscular tremor produce smaller, very irregular vibrations in the string shadow.

2. **Deformities due to changes in skin resistance and polarization of the electrodes.** These are characterized by a more gradual undulating shifting of the base line.

3. **Deformities due to improper grounding of the electrocardiograph and interfering induction currents.** These artefacts are characterized by regular vibrations due to 60 cycle alternating current at a rate of 60 times a second.

4. **Deformities due to a loose string.** If the resistance of the skin is too high, the string must be loosened to an unusual degree in order to standardize it. This prevents the string from stopping abruptly when a millivolt of current is added to the circuit so that the string ''overshoots'' or deviates above or below the proper amplitude. This would result in abnormally high amplitudes, slurring, and widening. When this occurs, the electrodes must be reapplied. The skin must be prepared more thoroughly, using electrode jelly to reduce skin resistance further. Overshooting must be watched for when taking each lead of the string galvanometer instrument.

5. **Deformities due to loose contacts.** Loose contacts at any place in the circuit may also cause sudden shifting of the base line.

Measurements of Amplitude, Heart Rate, Intervals, and Duration of Complexes

The duration of the P wave is measured from its convex surface at the instant it rises or falls from the base line to the moment it returns to this level. The duration of the QRS complex is measured from the beginning of the Q wave on its convex surface to the end of the R or S wave at its convex surface. The convex surface is used for making all other interval measurements such as P-R or P-Q, Q-T, and T.

The amplitudes of the upright deflections are measured from the upper surface of the base line to the upper surface of the topmost portion of that wave which is being measured. The inverted waves are measured from the lower surface of the isoelectric level to the apex at its lower surface. Duration and voltage of a complex are accepted as that measured in the lead in which they are the greatest.

Many ways of estimating the rates of the ventricles or auricles have been described. A convenient method is to count the number of time lines lying in the space of four cycles, either auricular or ventricular. Then 6000 is divided by this figure; the result gives the rate of the auricles or ventricles, according to which complexes were measured. Since the time lines are divided into groups of five by each heavy line (see above), it is convenient to begin measuring the four complexes where the first one falls on a heavy time line and then to count the heavy time lines, each representing groups of five, until the fourth complex is reached, and figure accordingly.

THE ELECTROCARDIOGRAM IN DISEASE AND IN DISORDERED PHYSIOLOGIC STATES

The electrocardiogram can be altered in many respects both by disease processes localized to the heart itself, and by general constitutional disorders. While many of these disease states cause electrocardiographic alterations which are nonspecific for that disease, there are others which are pathognomonic or highly suggestive of disease. The picture of acute myocardial infarction, for example, is usually characteristic. The location of this lesion can often be ascertained with a high degree of accuracy from the electrocardiogram alone. On the other hand, degenerative changes involving the myocardium, general constitutional disease, toxic agents, and even psychogenic factors may produce alterations in the electrocardiogram which are indistinguishable from each other.

The most significant changes in the electrocardiogram are those involving the QRS and T waves; in other words, those involving ventricular contraction. The size, shape, direction, and duration of the complexes depend on several factors. The relative thicknesses of the right and left ventricular muscles, for instance, determine whether the direction of the main deflection of QRS is up or down in Leads I or III. Certain diseases of the cardiovascular system alter the relative thickness of the right and left ventricles in characteristic fashion. The origin of the impulse which the muscle, auricular or ventricular, receives also determines the size, shape, and duration of the complexes. If, for instance, the impulse stimulating the auricles to contraction arises in the auricle in a point other than the normal location, the P wave will be altered in direction, size, or shape, and in its time relationship to QRS as well, since the time necessary for the impulse to reach the ventricles will be altered. It is normal for the impulse stimulating ventricular contraction to arise from a point above the ventricular musculature proper. As long as this obtains, the QRS and T complexes maintain a form, size, duration, and direction that is usual for this patient. If, however, a stimulus should arise in the ventricular muscle itself, the shape, size, duration, and direction are altered in a bizarre fashion, depending on the location of the exciting impulse in the ventricles. The T waves alone may be altered by multitudinous causes ranging from drinking cold water, psychogenic influences, or drug effects to serious organic disease involving the ventricular muscle. Any condition involving the pathway of conduction from the auricles to the ventricles may increase the duration of the P-R or P-Q interval.

In the same way, any disease or toxic process which impedes the conduction of the impulse as it spreads through the ventricles will cause increased duration of the QRS complex. The level and form of the S-T segments may be altered in certain characteristic respects by disease or toxic influences. Disordered rhythms of the heart, usually involving abnormal sites of impulse formation, and occurring as a result of a wide variety of organic and functional disturbances of the heart, give characteristic electrocardiographic patterns.

It is not our purpose here to give in detail the many patterns of electrocardiographic alterations that may occur. The electrocardiogram in the main types of cardiovascular diseases only will be discussed. An understanding of the pathologic physiology of disease is necessary in order to interpret abnormalities of the electrocardiogram.

Disturbances in depolarization and repolarization of heart muscle are caused by numerous factors both physiologic and pathologic; often these are manifested merely by lowering or inversion of the T wave. This change in T wave may not be specific for any type of lesion and may be caused by factors ranging from psychogenic influences to fibrosis resulting from long-standing and progressive coronary occlusion. In many cases it is possible to gain valuable clues to the nature of the disease by making serial electrocardiograms. With certain physiologic states, toxemias, and drugs, the T wave changes are reversible; with organic disease, such as fibrosis of the myocardium, the changes are permanent. Often when serial electrocardiograms are taken, the electrocardiograms undergo characteristic pattern changes which are pathognomonic of acute myocardial infarction. These changes involve the S-T segment and T wave primarily, and the QRS less strikingly.

Clues to the types of disease may be obtained if one understands the effects of disease in causing strain on the various chambers of the heart. Strain on the left ventricle, on the right ventricle, and on the auricles, with the consequent increase in muscle mass, produces characteristic evidence in the electrocardiogram. Some diseases or drugs are likely to exert their effects primarily on the conduction system, so that the presence of increased P-R conduction time will often give a clue to the disease or drug involved.

Axis Deviation.—Many cardiac diseases involve changes in relative size of the right and left ventricles with consequent effect on the electrical axis of the heart. This electrical axis may also be altered by changes in the position assumed by the heart in the chest cavity. Thick chested, sthenic individuals, with diaphragms that tend to be high, often have hearts that lie transversely in the thorax, whereas slender individuals with long chests often have hearts that assume a more nearly vertical position with consequently a different electrical axis with respect to the conventional leads. Since deep inspiration alters the position of the heart it therefore affects the electrical axis. This applies to changes in position of the heart occasioned by extracardiac lesions such as pneumothorax, fluid in the chest, pulmonary collapse, fibrosis with pull on the mediastinal structures, etc.

It thus becomes important to determine alterations in the normal electrical axis of the heart. When the largest deflection of QRS is upright (R) in Lead I and invert (S) in Lead III, this indicates the presence of left axis deviation. See Fig. 736. When the largest deflection of QRS is upright (R) in Lead III and invert (S) in Lead I, right axis deviation is present. See Fig. 737. Left axis deviation is present in any condition that causes relative enlargement of the left ventricle over the right.

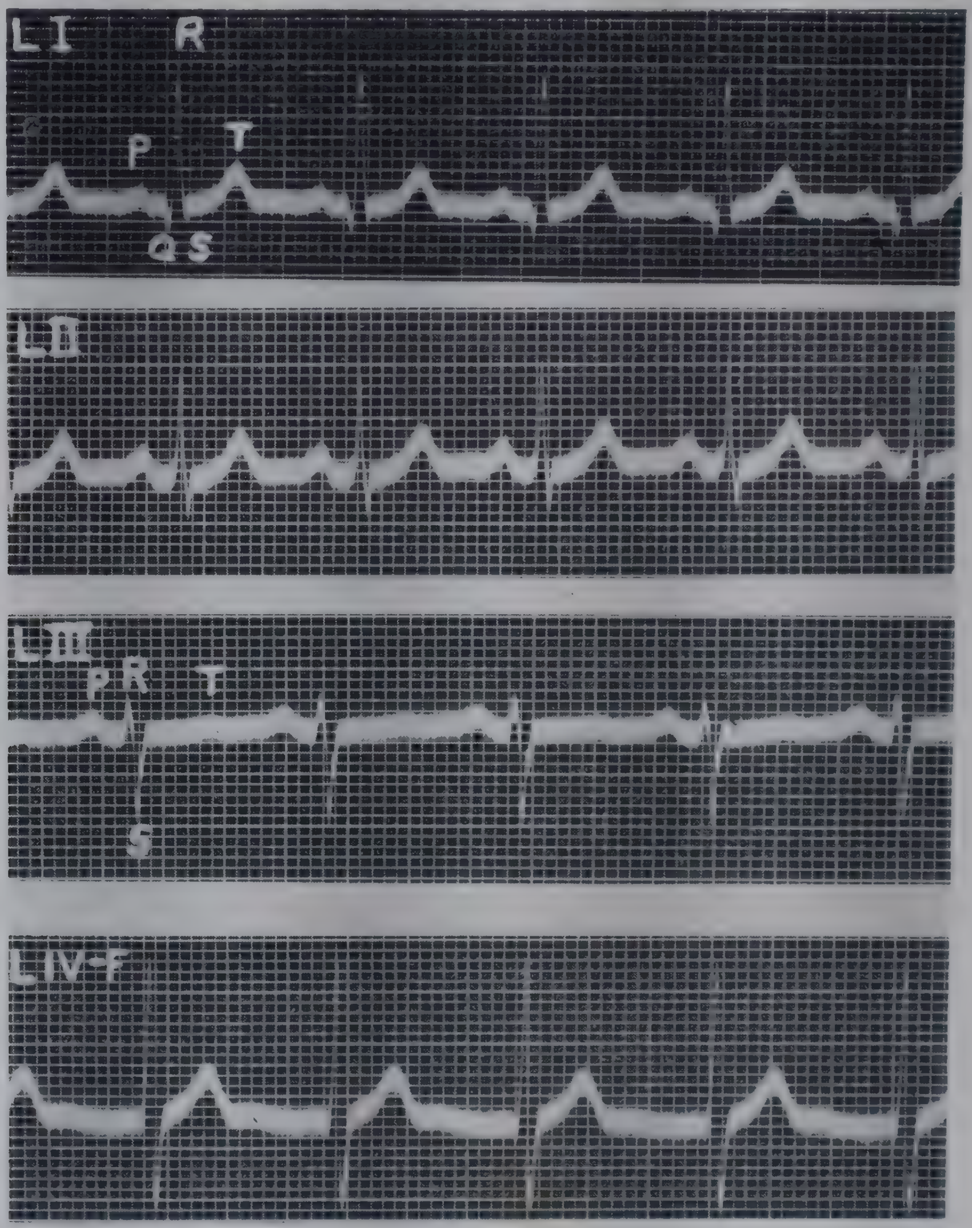


Fig. 736.—Left axis deviation. Note that the chief deflection in Lead I (R) is upright and the chief deflection in Lead III (S) is inverted. A small R is present in Lead III. In more marked degrees of left axis deviation R in Lead III may be entirely absent.

Right axis deviation occurs when there is relative enlargement of the right ventricle over the left. A transversely placed heart in which the apex lies more to the left produces the picture of left axis deviation. A vertically placed heart tends to produce a picture approaching right axis deviation. In young children, when the right ventricle is relatively thicker in relation to the left, the picture more nearly approaches right axis deviation, but, with age, as the left ventricle thickens, the picture of left axis deviation may finally be produced.

Deep inspiration will often cause a deeply inverted deflection (S) in Lead III to disappear, and abolish the picture of left axis deviation by producing a vertical position of the heart. If the right and left ventricles both enlarge and maintain their normal relationship with respect to size, then no change in electrical axis may occur. The occurrence of axis deviation can be helpful in determining the presence or absence, and the type, of valvular disease, since it is known that certain types of valvular disease tend to produce right axis deviation, whereas others tend to cause left axis deviation. The characteristic murmurs of these valvular deformities, or of congenital anomalies as well, may be absent or confusing; the finding of axis deviation suggestive of one or the other valvular condition may be decisive in the diagnosis. Changes in axis deviation associated with various types of cardiac pathology are mentioned in the discussion of electrocardiographic patterns found in the main classes of cardiac

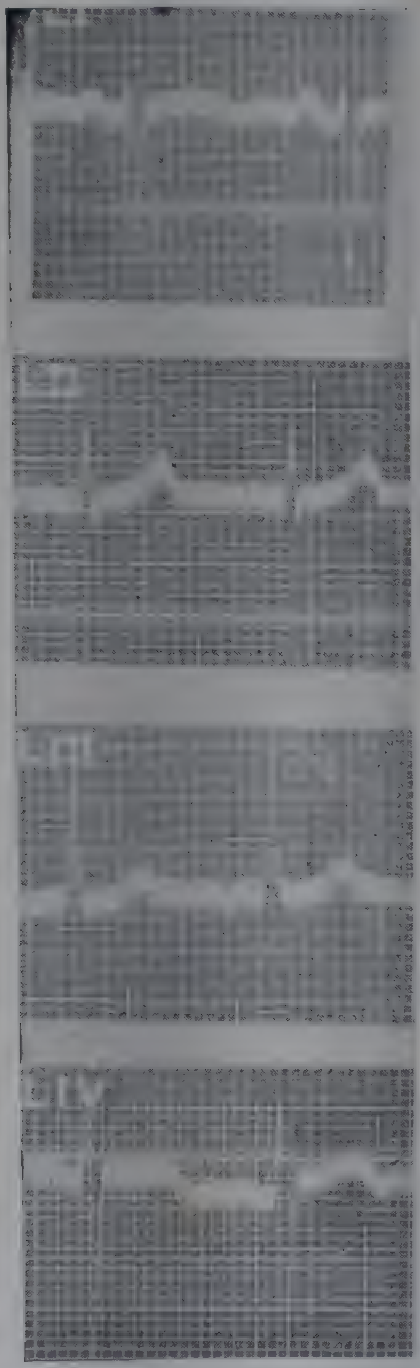


Fig. 737.—Right axis deviation. Note that the chief deflection (S) is down in Lead I and up (R) in Lead III. The R is small in Lead I but may be entirely absent.

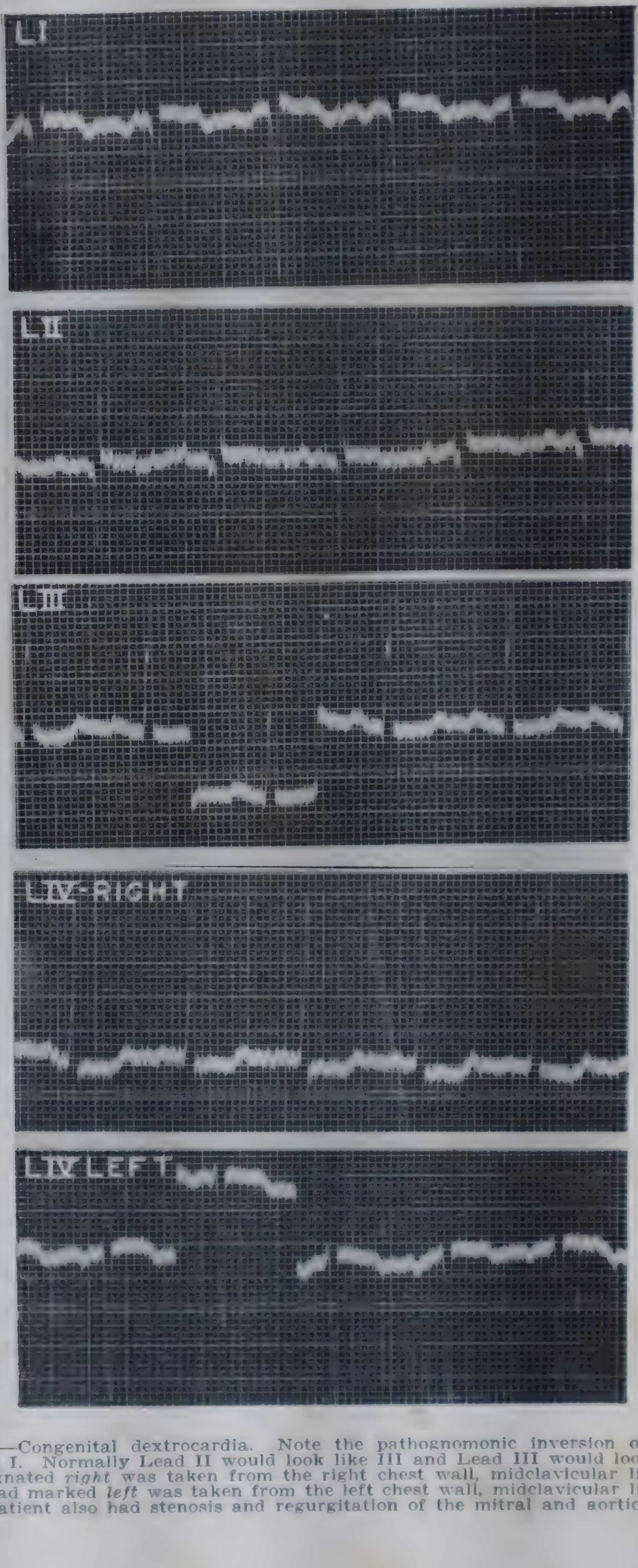


Fig. 738.—Congenital dextrocardia. Note the pathognomonic inversion of all the complexes in Lead I. Normally Lead II would look like III and Lead III would look like Lead II The lead designated *right* was taken from the right chest wall, midclavicular line—fifth interspace. The lead marked *left* was taken from the left chest wall, midclavicular line—fifth interspace. This patient also had stenosis and regurgitation of the mitral and aortic valves.

diseases. It is well to emphasize that left axis deviation may often be a normal occurrence. In these instances T_1 is upright, T_3 inverted, and S_{II} is absent or very small.

There are several methods for calculating the electrical axis of any electrocardiogram. The three most commonly used are: (1) Einthoven's triangle; (2) the triaxial reference system; and (3) Dieuaide's chart. These methods are well described in monographs devoted to electrocardiography and its underlying theories.

Congenital Heart Disease

The most striking and characteristic electrocardiographic changes occurring in congenital heart disease are those found in true congenital dextrocardia (right-sided heart). In this condition all the complexes in Lead I are inverted, Lead II resembles a normal Lead III, and Lead III resembles a normal Lead II.

Diagnosis of congenital dextrocardia may be made with absolute certainty from the electrocardiogram alone if the electrocardiographer can be sure that the technician who took the tracing did not reverse the arm leads, erroneously connecting the right arm lead wire to the left arm electrode, and the left arm lead wire to the right arm electrode. Unfortunately this careless technical error is by no means a rare occurrence. It behooves all those who are responsible for making electrocardiograms to check carefully all lead wires several times to make sure that no such errors will be made.

Other congenital anomalies which commonly result in electrocardiographic changes are congenital pulmonary stenosis and defective interventricular septum. These conditions cause well-marked right axis deviation. The QRS complexes are of much greater amplitude than usual. Congenital heart block may occur in which the auricular complexes (P) have no constant relationship whatsoever to the QRS complexes; in other words, the ventricles contract independently of the auricles because the stimulus from the auricles is blocked off somewhere in the conduction pathway from the auricles to the ventricles.

Rheumatic Heart Disease

Rheumatic fever may cause changes in the endocardium, chiefly the valves, the myocardium, or the pericardium; or changes may occur in all these structures at the same time. One of the most characteristic electrocardiographic effects of rheumatic fever in its acute stage is involvement by the acute inflammatory process of the auriculoventricular conduction system. This results in delay in auriculoventricular conduction with a subsequent increase in the P-R interval. This depression in conduction with consequent increase in P-R interval may be very slight; or it may become so marked that the ventricles do not receive any impulse at all from the auricles. Thus they do not contract in response to auricular stimulation so that one may see a P wave (auricular) without its corresponding QRS complex.

These varying degrees of increased P-R interval represent what is known as *heart block*. Heart block may be so marked as to be complete. If the block is complete, no ventricular complexes occur in response to the auricular wave. In complete heart block each of the ventricular complexes occurs independently of the auricular waves at a much slower rate than the latter. Milder degrees of heart block, in which each ventricular contraction responds to an auricular contraction, cannot be detected by ordinary clinical means. The electrocardiograph is invaluable in these conditions. It gives definite, objective, confirmatory evidence of the presence of acute rheumatic fever. Usually such P-R interval changes in acute rheumatic fever are transitory and disappear when the acute inflammation subsides.

The inflammatory changes of rheumatic fever which involve the myocardium proper cause alterations in the electrocardiogram not so specifically associated with rheumatic fever as the P-R interval changes. Other types of pathologic processes may also cause these patterns. Such manifestations may be characterized by slurring, or notching, lowering or widening of the QRS complex, lowering or inversion of the T waves, and changes in the Q-T intervals.

If the pericardium is involved by the rheumatic inflammatory process there will likely be elevation of the S-T segments in Leads I, II, and IV F. These changes are not specific for rheumatic fever, since any process which alters depolarization by involving the pericardium in this way can cause such electrocardiographic patterns.

The electrocardiographic pattern which develops with chronic endocarditis and consequent valvular involvement depends upon the valve involved. Since the mitral valve is frequently stenosed, increased strain is placed on the chambers behind this valve; namely, the left auricle, the right ventricle, and eventually the right auricle. The pattern of right axis deviation develops with increased right ventricular strain and increased muscle mass. Because the left auricular muscle so often becomes hypertrophied in mitral stenosis the P wave is altered, becoming prominent and notched. The auricular muscle is increased and a longer time is needed for the action current to traverse it; thus there is widening of the P wave. This type of P wave, characteristically present in Leads I and II, is quite suggestive of mitral stenosis.

A disorder of rhythm, known as auricular fibrillation, is very common in rheumatic heart disease with mitral stenosis because of the strain upon the left auricle.

If the mitral valve involvement is such that regurgitation is prominent and the stenosis is relatively minor or absent, strain is put upon the left ventricle, and left axis deviation occurs. This is not so suggestive of rheumatic heart disease since many more pathologic heart conditions other than rheumatic heart disease may give left axis rather than right axis deviation. If the aortic valve is involved and causes regurgitation or stenosis of that valve, a frequent occurrence in rheumatic heart disease, strain is put primarily on the left ventricle. This ventricle hypertrophies and left axis deviation develops. If both mitral stenosis and aortic stenosis or aortic regurgitation are present, and if both right and left ventricles hypertrophy, then the electrical effects of right and left ventricular hypertrophy may counterbalance each other and no axis deviation may result.

Hypertension

In hypertension, especially of a marked degree and of long standing, a rather typical electrocardiographic pattern of left ventricular strain develops. The R wave in Lead I is usually tall, the S-T segment becomes depressed and coved in Lead I and elevated in Lead III. The S wave is deep in Lead III, and the R wave is relatively small or absent in Lead III. The T waves in Leads I and III are in opposite direction to those of the main deflection of QRS in Leads I and III; that is, inverted in Lead I and upright in Lead III. The QRS may be slightly widened in some instances due to the additional time required for the action current to traverse the thickened ventricular muscle. Fig. 739 is an example of this type of pattern.

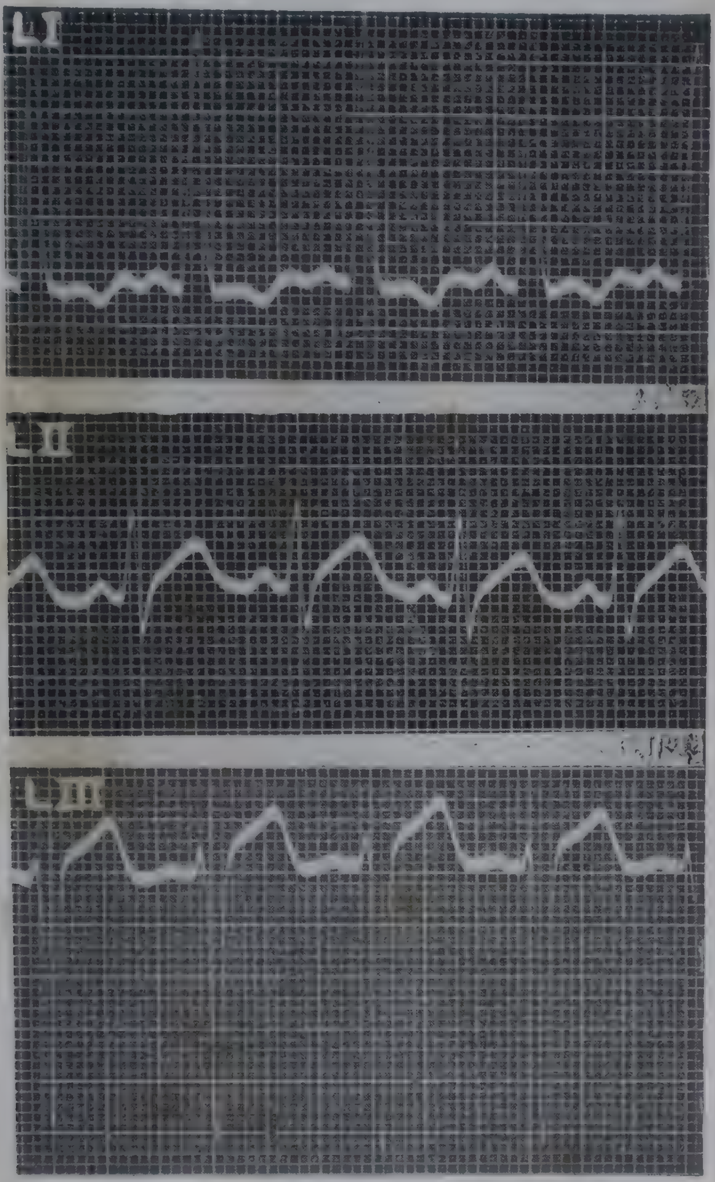


Fig. 739.—Left ventricular strain in hypertension. Note the tall R wave in Lead I, the deep S wave in Lead III; also the depression of the S-T segment in Lead I, and the elevation of this segment in Lead III. The T is inverted in Lead I and upright in Lead III. Note also the slightly increased duration of QRS which is due to the increased time necessary for the impulse to traverse the thickened ventricular muscle mass.

Myocardial Infarction

The electrocardiograph has been of great value in ascertaining definitely the presence of myocardial infarction. This condition is commonly referred to as **coronary thrombosis**. Since, however, the electrocardiographic patterns are caused directly by resulting actual necrosis of the heart muscle itself, that is, the infarction of the muscle, and not by the thrombus which obstructs the coronary artery, it is more correct to refer to these electrocardiographic changes as being due to myocardial infarction. This fact must be emphasized that, while most cases of myocardial infarction are due to coronary thrombosis, *occasionally myocardial infarction occurs in cases with extensive narrowing of the coronary arteries without thrombosis.*

The electrocardiographic picture of myocardial infarction varies according to the site and extent of the coronary artery obstruction. In general, there are two main varieties of coronary obstruction, anterior and posterior. These pictures may be modified by such factors as presence of previous infarctions, involvement of the conduction system, or involvement of the pericardium or endocardium.

Anterior Myocardial Infarction.—Most commonly the left anterior coronary artery is obtructed, with resultant infarction of the anterior apical portion of the left ventricle. If serial electrocardiograms are then taken, a characteristic series of changing electrocardiographic patterns will be produced. The QRS complexes in Lead I are frequently small, slurred, and W shaped. The earliest (within the first two days) and most characteristic change is an elevation and upward convexity of the R-ST segment in Lead I and a reciprocal depression of the same segment in Lead III. The T wave early has a high take-off from the S-T segment and may have merely a plateau type of configuration with perhaps a slight terminal dip. Later the S-T segment in Lead I drops steadily until it assumes a position at or slightly below the isoelectric level, maintaining a characteristically upward convexity.

The T wave in Lead I becomes completely inverted with a characteristic ''coved'' take-off from the S-T segment. The opposite changes occur in Lead III. The Q wave in Lead I, which has made its appearance early, will usually persist. The S-T segments and T waves very occasionally may eventually resume a completely normal pattern. The characteristic residual patterns of an anterior myocardial infarction are the prominent Q and inverted and coved T waves in Lead I, with opposite configuration (normal) in Lead III. This is known as the Q_IT_I pattern of anterior myocardial infarction.

Changes in the chest lead (IV F) in anterior infarction are quite characteristic, and frequently even more striking than in the other leads. Occasionally, in this type of infarction, Lead IV F may be the only lead which reveals the changes characteristic of anterior infarction, thus emphasizing the value of this lead in this condition. In general, the changes in Lead IV F parallel those in Lead I except that they are often more marked. In the early stages the S-T segment is displaced upward and the initial deflection of QRS may be only very slightly upright or deeply inverted (deep Q). A deep Q in Lead IV F is never normal and usually signifies coronary artery obstruction. The T in the early stages has an elevated take-off from the S-T segment which is short and abrupt, and often exhibits a slight terminal dip. Gradually, the S-T segment becomes depressed until it reaches the isoelectric level or a point slightly below it.

The T wave becomes sharply and deeply inverted. The Q wave is characteristically prominent and is more definitely indicative of the presence of anterior

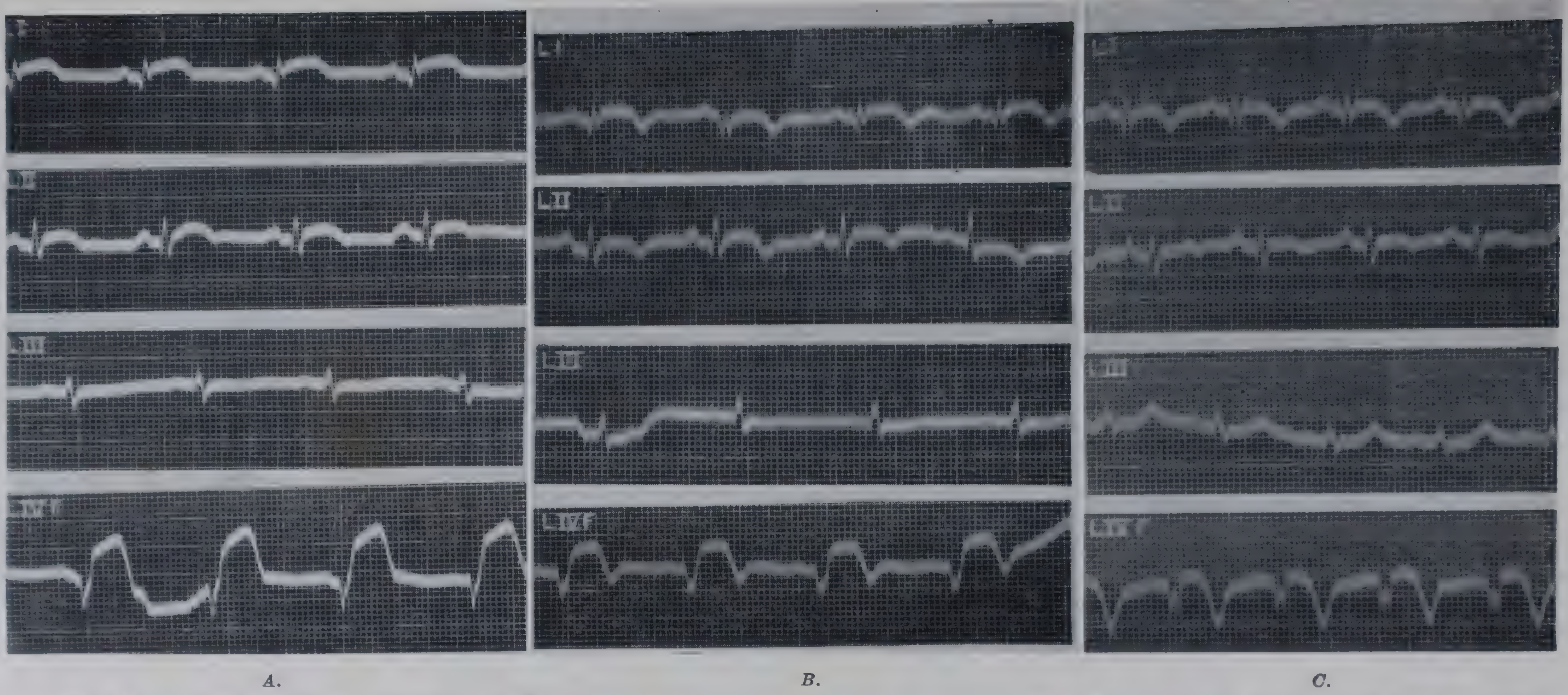


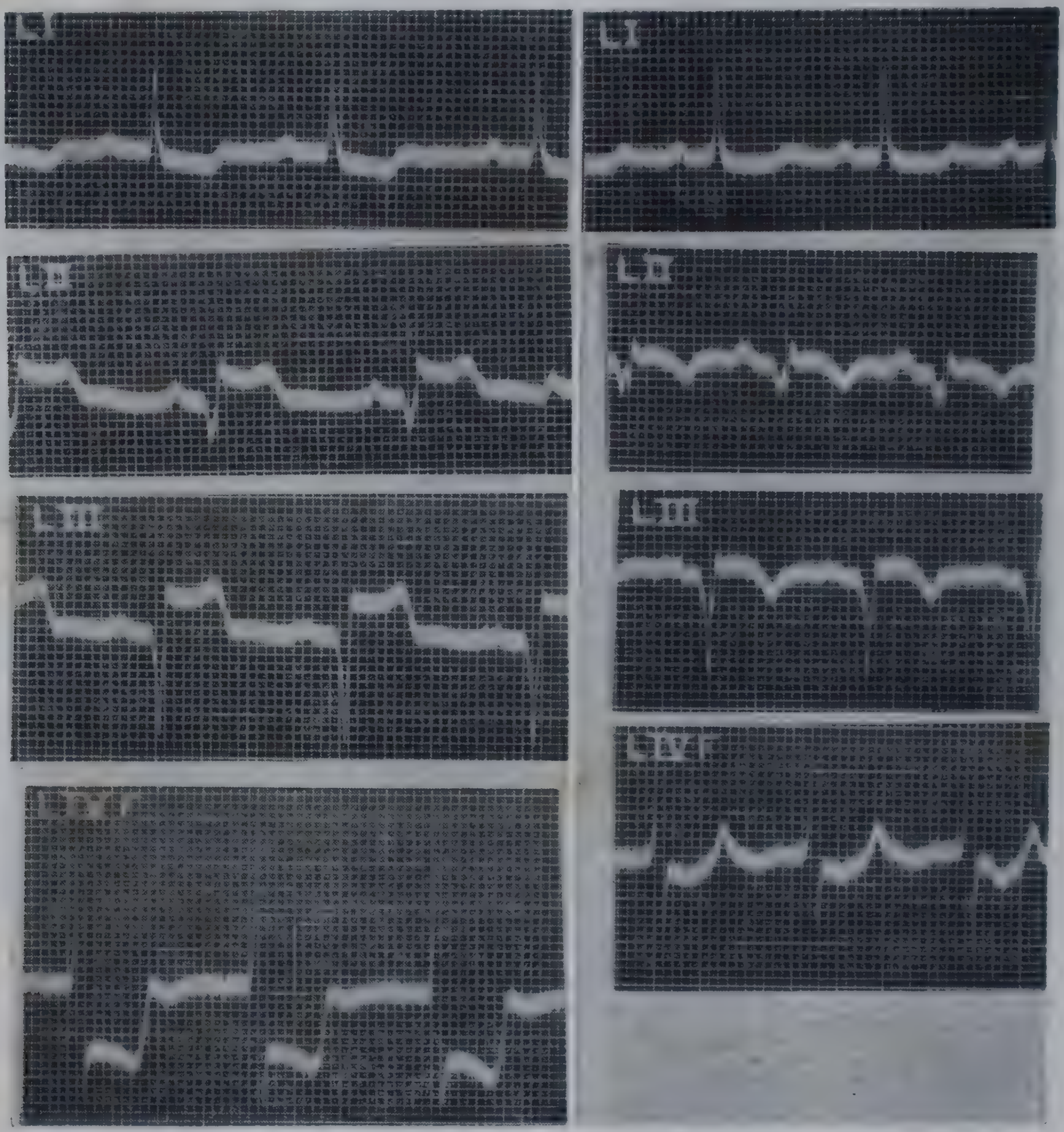
Fig. 740.—Anterior myocardial infarction. *A*, Electrocardiogram taken the day of the attack. The S-T segment is elevated and has an upward convexity in Leads I and II; in Lead III the S-T segment is slightly depressed, whereas in Lead IV F there is marked elevation and upward convexity of the S-T segment. A small Q wave is present in Leads I and II while in Lead IV F the entire QRS is inverted and notched—no R is present.

B, Taken four days after *A*. Note the lowering of the S-T segment with less upward convexity in Leads I, II, and IV F. The T waves in Leads I, II, and IV F are now definitely inverted. The Q waves in Leads I and II persist; R wave remains absent in Lead IV F.

C, Taken 28 days after *B*, 32 days after onset of illness. Note further lowering of the S-T segments in Leads I, II, and IV F almost to the isoelectric level. There is further deepening of the inverted T wave in Leads I and IV. T wave in Lead II is less inverted as would be expected with the appearance of the upright T wave in Lead III. Note also the sharply rounded convexity of the S-T segment in Lead IV F—the "coving" so characteristic of myocardial infarction. The QRS complex is W-shaped in Leads I, II, and IV; in Lead IV the entire QRS complex is below the isoelectric level.

infarction than an inverted T wave. Nevertheless a deep Q wave might never appear in Lead IV F, and an inverted T wave in Lead IV F may be the only evidence of an anterior myocardial infarction.

If the initial deflection in Lead IV F is upright (R IV) but is only 2 mm. or less in amplitude, it is quite suggestive of the presence of myocardial infarction. If an inverted T wave as well is found, then the evidence of myocardial infarction is more nearly conclusive. Fig. 740 shows the sequential changes, over a period of several weeks, in a case of acute anterior myocardial infarction.



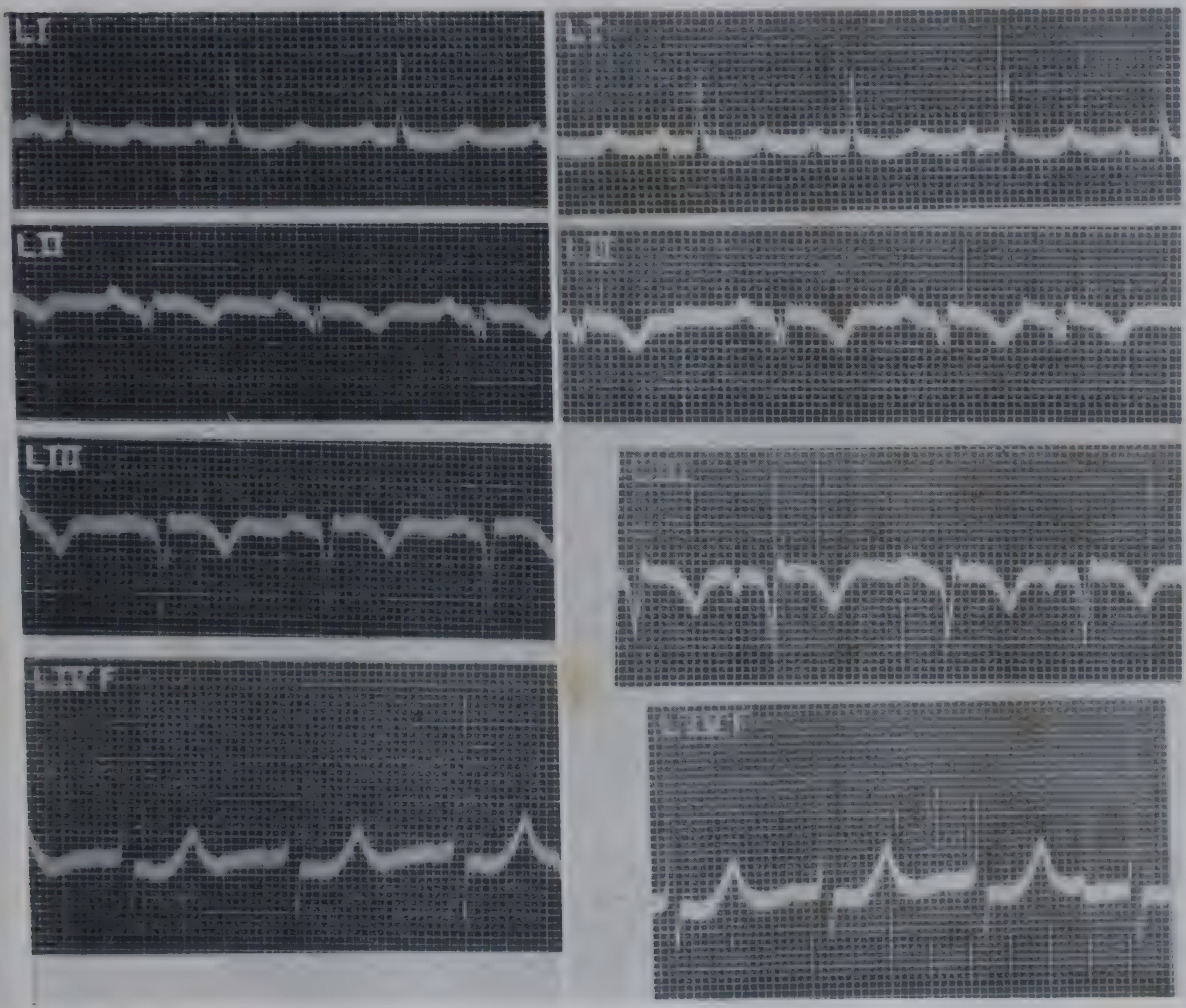
A. *B.*

Fig. 741.—Posterior wall myocardial infarction. Showing typical serial changes from the first day to the twenty-fifth day after the attack.

A, Shows most marked changes in Leads II, III, and CF_{IV}. In Lead I the S-T segment is slightly depressed whereas in Leads II and III the S-T segments are markedly elevated and plateau type. In Lead CF_{IV} the S-T segment is markedly depressed and has an upward convexity. The T wave in Lead I is diphasic and in Leads II and III has a high take-off with a slight upright direction. In Lead CF_{IV} the T wave has a low take-off and is diphasic. Note the characteristically prominent Q waves in Leads II and III.

B, Taken 15 days after onset. Note that the S-T segments in all the leads are returning toward the isoelectric level. The S-T segments in Leads II and III show a characteristic upward convexity, "coving." The T waves are now completely inverted and still show a slightly elevated take-off from the S-T segments. The Q waves remain prominent and notched in Leads II and III. The T wave in Lead CF_{IV}, while still showing a rather low take-off, is now almost completely upright.

Posterior Myocardial Infarction.—Posterior myocardial infarction shows changes similar to those of anterior infarction in Leads I and III except that the patterns are reversed. The characteristic rise in the R-S-T level takes place in Lead III and usually in Lead II, while reciprocal depression occurs in Lead I and often in Lead IV. A prominent Q wave in Lead III and often in Lead II becomes evident just as in anterior infarction a Q_I makes its appearance. There is the same upward rounding or convexity of the S-T segment, together with the elevation of this segment, the same stages of progressive lowering of S-T segment and inversion of T wave in Lead III as a result of acute posterior infarction as are found in Lead I in cases of anterior infarction. Variations from these classical patterns are seen. These depend upon the presence of the infarction in locations other than those described above; or they may be due to the fact that an old lesion has preceded the fresh lesion.



C. *D.*

Fig. 741.—*C*, Taken 18 days after onset. No significant change over the electrocardiogram taken three days earlier.

D, Taken 25 days after onset. Still shows no further significant change over the tracing taken one week before. The electrocardiogram has become stabilized in the typical pattern of posterior wall infarction characterized by prominent Q_{II} and Q_{III}, and inverted and coved T_{II} and T_{III}. Alterations in Leads I and IV often are not present or when present are minimal and in opposite direction to those of Leads II and III.

In the chest lead (IV F) changes with posterior infarction are not striking. The S-T segment may be depressed to varying degrees and may gradually rise to the isoelectric level. The T wave is normal or is occasionally unusually tall. Fig. 741 shows the serial changes occurring in a case of acute posterior myocardial infarction.

More information relative to posterior infarction may be obtained by using esophageal leads since this lead is placed as near the posterior surface of the heart as it is possible to place a lead in a living subject.

Syphilitic Heart Disease

The characteristic lesion of syphilitic heart disease is aortic regurgitation. Since changes in an electrocardiogram depend on muscular involvement and not on valvular defects, the effect of aortic regurgitation on the electrocardiogram will be caused by secondary results of this valve lesion, namely, left ventricular enlargement. As stated above, increase in size of the left ventricular muscle causes left axis deviation. It is thus evident that the electrocardiographic pattern which one expects for the usual syphilitic involvement of the cardiovascular apparatus is not specific for that condition. It may occur as well in any other condition causing left ventricular enlargement. If the syphilitic aortic regurgitation is early or is only slight, and no significant left ventricular enlargement has occurred, the electrocardiogram may then show a normal axis. Other changes (nonspecific) in the electrocardiogram may occur if the mouths of the coronary arteries are obstructed by a syphilitic lesion of the aorta with resultant fibrosis of the myocardium. The electrocardiographic effects will then be not unlike those of chronic coronary obstruction from other causes—inversions of T waves, alterations in conduction, spread and notching of QRS complexes, and increased P-R interval.

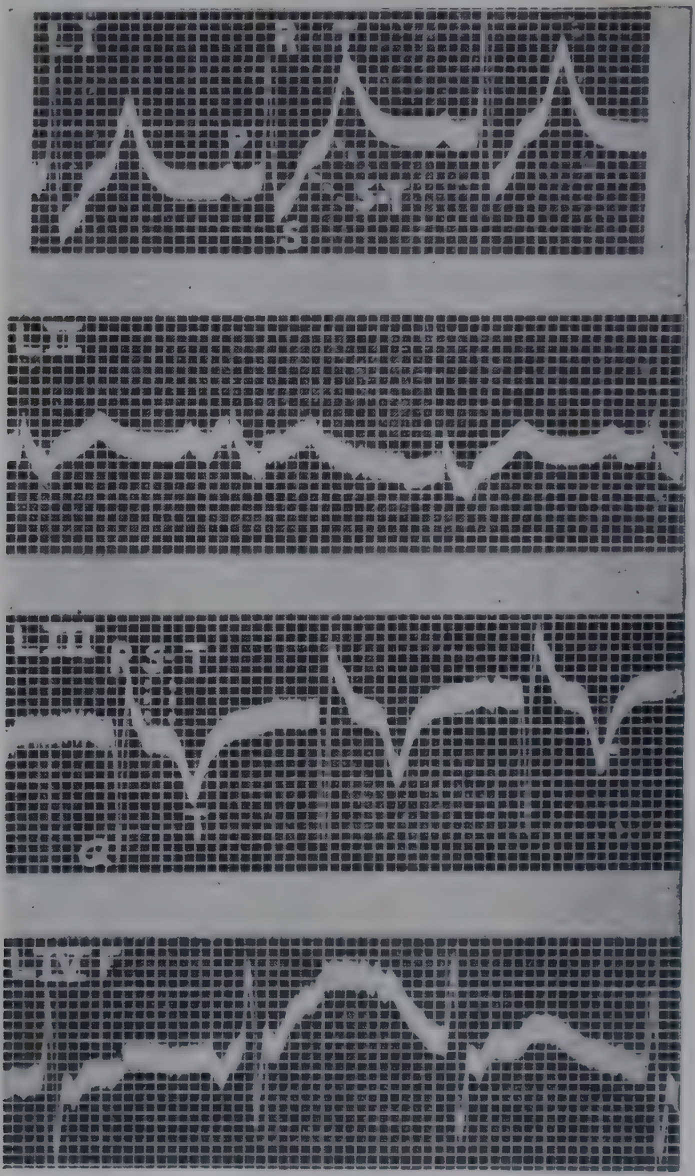


Fig. 742.—Acute massive pulmonary artery obstruction. Note the prominent wide and slurred S wave and the short steplike S-T segment in Lead I. The deep Q wave and inverted T wave in Lead III resemble those of posterior wall myocardial infarction.

Acute Pulmonary Artery Obstruction

Acute pulmonary artery obstruction is a condition which is often catastrophic and may cause death instantly or within a short period of time. The electrocardiogram often gives characteristic patterns which will fortify the physician in his decision to take heroic measures for treatment. It should be emphasized, however, that the electrocardiogram may be entirely normal in this condition.

The S wave in Lead I is prominent and wide, and leads rather abruptly and by a steplike configuration of the shortened S-T segment into the T wave. Q waves are often present in Leads II and III, and T_{II} and T_{III} are inverted Note that the pattern of Q_{III} and inverted T_{III} is that described for posterior infarction. The prominent, wide S_I and the characteristic S-T segment in Lead I, as well as the much more transitory nature of the picture in acute pulmonary artery obstruction, all help to differentiate it from posterior infarction where the changes endure for months and are usually permanent. The electrocardiogram of acute pulmonary artery obstruction often, though not necessarily, reveals right axis deviation because of the strain on the right heart.

Pulmonary Emphysema and Pulmonary Artery Sclerosis

Pulmonary emphysema and pulmonary artery sclerosis may cause changes in the electrocardiogram by increasing the strain on the right ventricle and thus result in right axis deviation. The P wave is often characteristically altered in pulmonary emphysema. It becomes tall and sharply peaked in Leads II and III (the P pulmonale) in contrast to the P wave of mitral stenosis, which is broad, prominent, and notched in Leads I and II. The T waves not infrequently may be low or inverted. Fig. 733 shows example of P pulmonale.

Any other conditions which affect oxygenation of the heart muscle, such as anemias, can be expected to cause lowering or inversion of T waves.

Thyroid Disturbances

Thyrotoxicosis is commonly associated with an electrocardiogram which shows merely normal complexes occurring at a faster than normal rate; that is, sinus tachycardia. Occasionally the QRS and T waves may show greater voltage than usual. One common abnormality of rhythm, which is manifested by hearts exhibiting the effects of the increased cardiovascular strain of thyrotoxicosis, is auricular fibrillation which is described below under "abnormal rhythms."

The opposite picture of thyrotoxicosis, myxedema, is often associated with electrocardiographic changes revealing low QRS and low or inverted T waves as well as slow rates (sinus bradycardia).

Deficiency Diseases

Deficiency diseases, of which beriberi is an outstanding example, often show electrocardiographic evidences of myocardial involvement such as low ventricular complexes (QRS and T).

Toxic States

Acute toxic conditions such as diphtheria and scarlet fever are examples of toxic diseases which may cause changes in T waves—flattening or inversion—and varying degrees of heart block—increase in P-R interval.

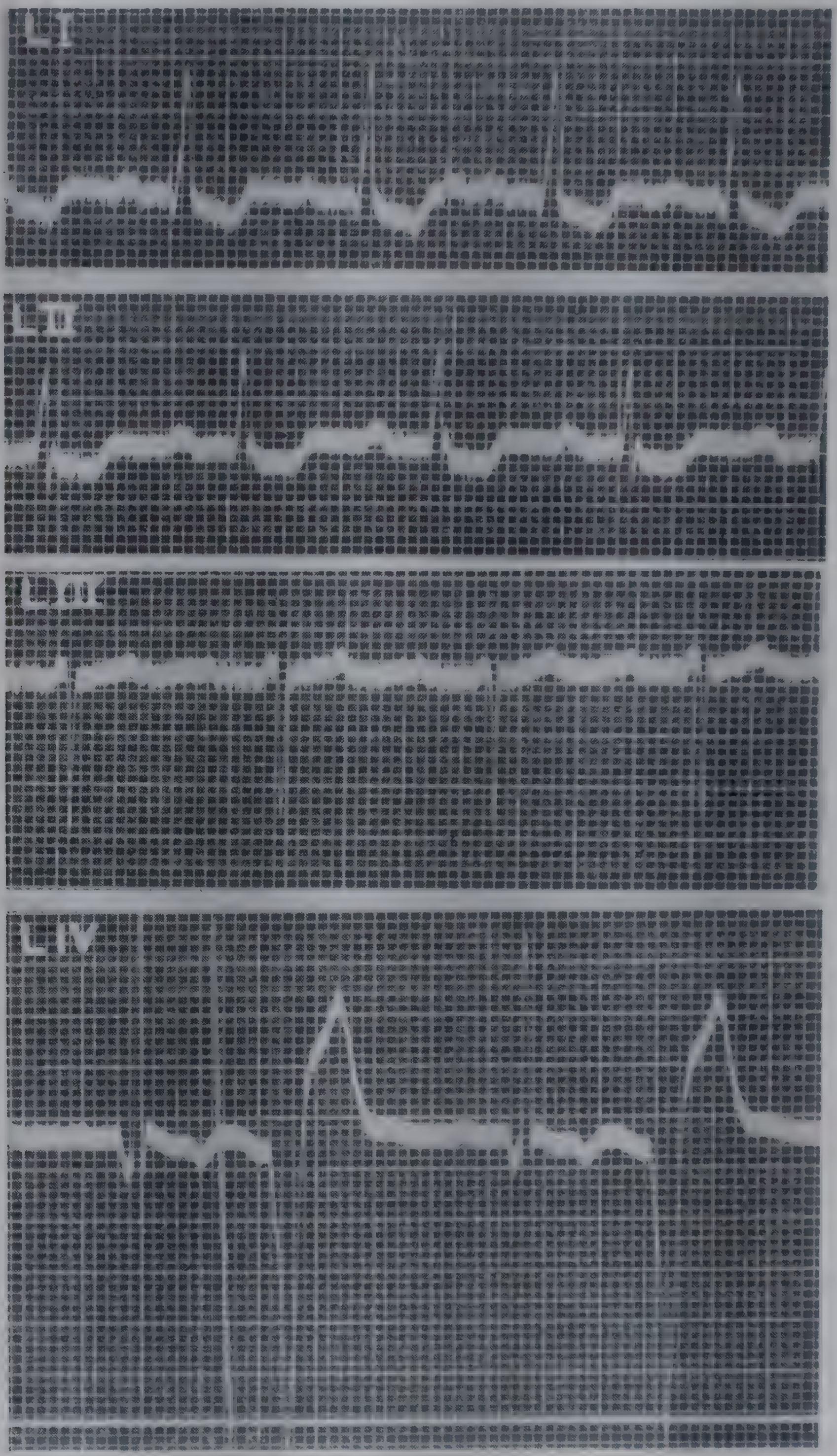


Fig. 743.—Digitalis effect. The R-T segments are depressed with abruptly downward deviation and no convexity upward. The T waves have become inverted in Leads I, II, and IV. The characteristic changes are here most typically shown in Leads I and II. In Lead IV are seen ventricular premature beats occurring every second cycle (pulsus bigeminus), a toxic manifestation of digitalis. The P-R interval is increased to 0.23 second, this being also a toxic effect of digitalis.

Chronic toxemias, such as uremia, often exhibit effects on the electrocardiogram. The QRS interval is often increased, the T waves inverted, the S-T segment depressed, and the Q-T interval increased as well. Pericarditis, a complication of uremia, may superimpose its effect upon the electrocardiogram.

Drug Effects

It is important to consider the effects of certain drugs on the electrocardiogram. Digitalis, which is the most important drug used in the treatment of heart disease, exerts certain characteristic effects. The most characteristic effect is on the S-T segment which descends abruptly without any tendency toward upward convexity as in the coronary S-T segments. The T waves are also depressed and eventually become inverted with full digitalis effect.

Digitalis also manifests an effect on auriculoventricular conduction so that the P-R interval is increased to the extent that even marked degrees of heart block are produced. In toxic effects many different types of disturbances of rhythm, most often extrasystoles, are produced. Fig. 743 shows the effect of digitalis on the electrocardiogram. Quinidine, another drug commonly used especially in certain arrhythmias, manifested by increased irritability of the heart muscle, depresses conduction and may produce spreading of the QRS, lengthening of the Q-T interval, and notching of the T wave. Morphine sometimes produces flattening or inversion of T waves.

Neurocirculatory Asthenia

The importance of distinguishing psychogenic effects on the electrocardiogram from those of organic diseases has been pointed out. These psychogenic effects are manifested by flattening, notching, or inversion of the T waves usually in Leads II, III, or IV, and very occasionally in Lead I. They have been often mistaken for organic effects. The importance of distinguishing between the changes in the electrocardiogram caused by neurocirculatory asthenia and those caused by organic heart disease is obvious since it involves matters affecting the happiness of the patient—prognosis, insurability, physical activity, desirability of marriage, etc. That such distinction can often be made has been pointed out by Wendkos, who restored abnormal T waves to normal by the use of certain drugs which act upon the autonomic nervous system.

In many patients either with or without organic heart disease, a great variety of disturbances in rate, rhythm, and conduction may occur. It is important to remember that these abnormalities in many instances do not necessarily indicate the presence of organic heart disease, but that, on the other hand, some are more likely to be associated with serious heart disease than others. It is not our purpose here to describe these clinical correlations, but rather to describe briefly those disorders in mechanism which are more commonly seen. Such uncommon mechanisms as parasystole and interference dissociation will not be discussed in this chapter.

NORMAL VARIATION IN RATE AND RHYTHM

In a normal electrocardiogram, the normal sequence of complexes, P, QRS, and T occurring in their correct relationships with each other and at a rate of 60 to 100 per minute, is known as a normal sinus mechanism. The impulse starts in its normal place in the sinus node and passes through the auricles down the A-V node, the bundle of His, the right and left bundle branches, and throughout the ventricle in a normal manner.

Sinus Tachycardia.—Impulses emanating from the sinus node occur at a rate exceeding 100 per minute. Because the impulse follows the normal conduction pathways the complexes are situated normally with respect to each other. The increased rate results in varying degrees of shortening of the T-P interval. If the rate is fast enough the T-P interval may be so shortened that P waves are superimposed upon the preceding T waves. Fig. 744 shows sinus tachycardia.

Sinus Bradycardia.—Here again the mechanism of conduction is normal and consequently the relationships of the complexes are normal. The rate, however, is below 60 per minute. Fig. 745 shows sinus bradycardia.

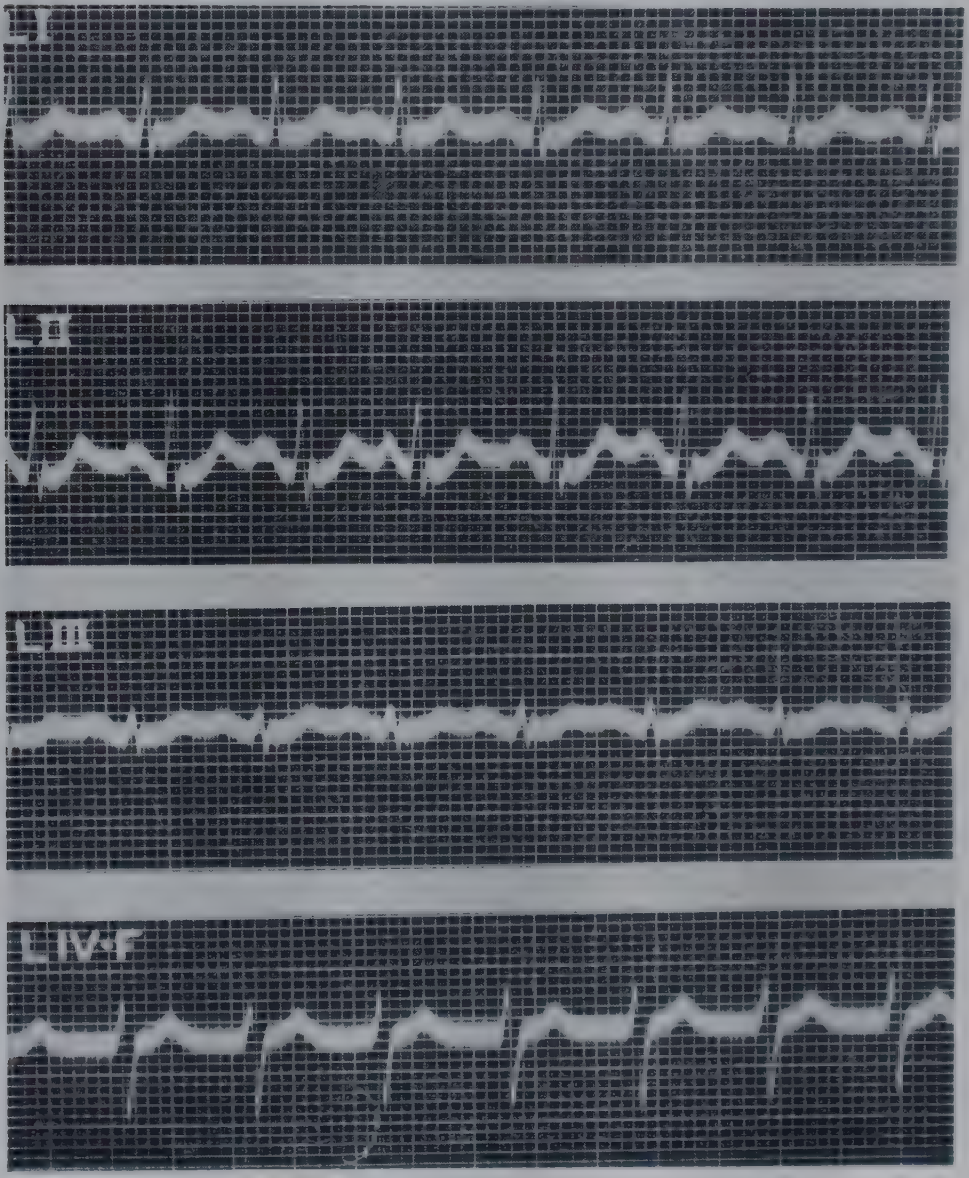


Fig. 744.—Sinus tachycardia. The configuration and duration of the complexes are within normal limits. The heart rate is increased to 139 per minute.

Sinus Arrhythmia.—The impulse for cardiac contraction originates in the normal place in the sinus node. However, because of variations in vagus nerve tone affecting the speed of impulse formation in the sinus node, the rhythm varies, with regularly recurring faster and slower cycles. The configuration of the complexes and the relationships between the auricular and the ventricular complexes are normal; the only variation is a regularly recurring increase and decrease of the T-P intervals with the slower and faster cycles. This mechanism is considered a normal alteration in rhythm. It depends, as a rule, upon the phase of respiration, an increase in rate occurring with inspiration and a decrease with expiration.

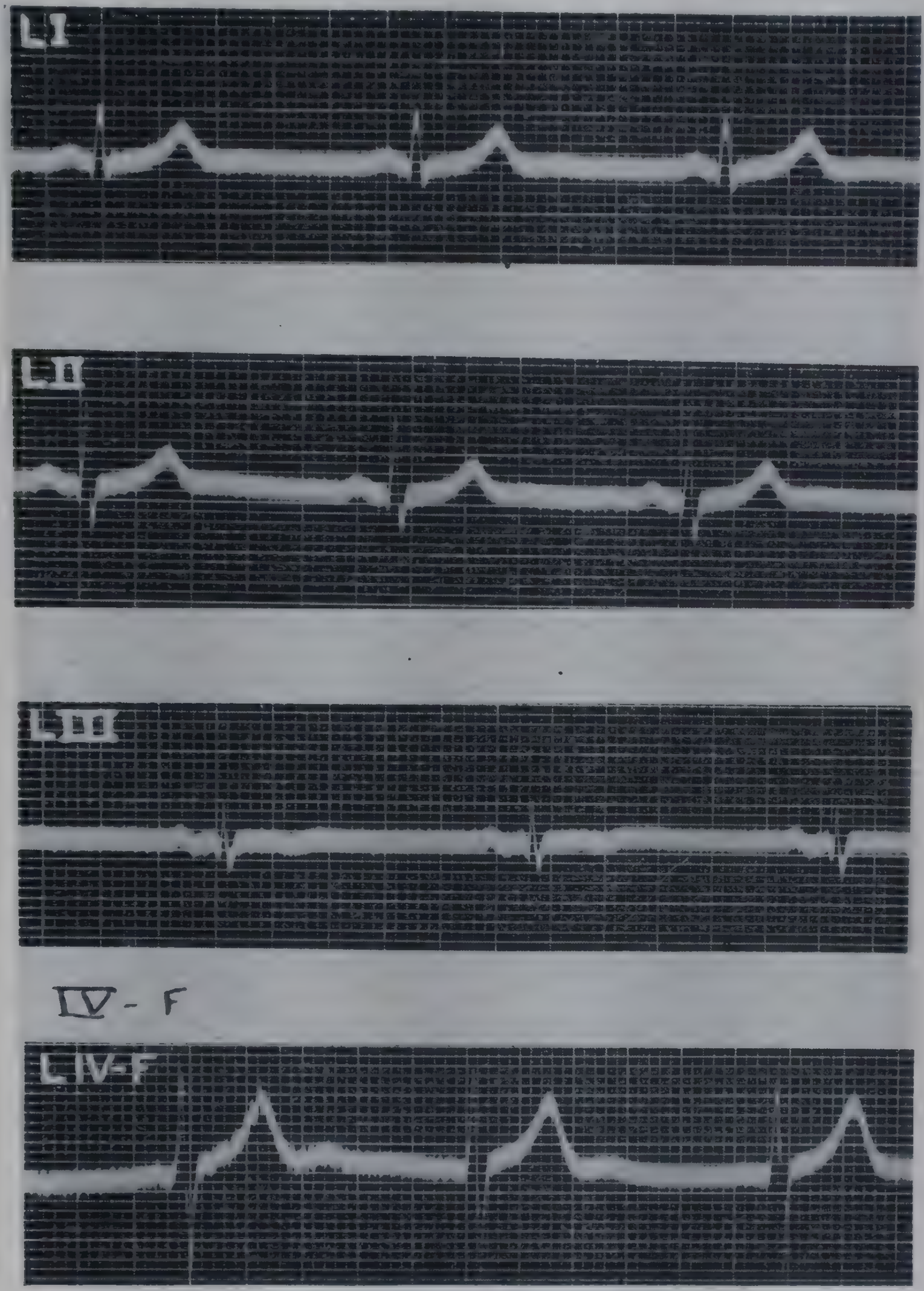


Fig. 745.—Sinus bradycardia. The configuration and duration of the complexes are within normal limits. The heart rate is 54 per minute.

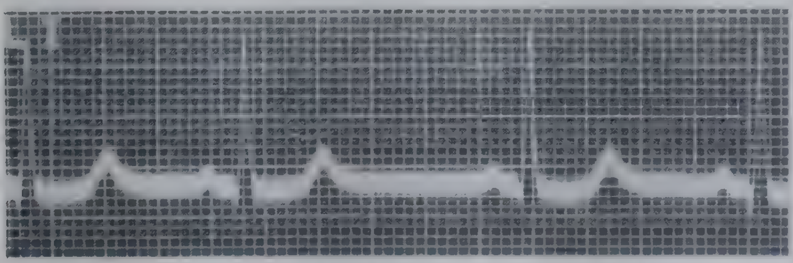


Fig. 746.—Sinus arrhythmia. Note that the auricular and ventricular complexes are of normal configuration and have normal time relationships. The only alteration is the shortening and lengthening of the T-P intervals.

ABNORMAL VARIATION IN MECHANISM

Disturbances in the Pacemaker.—Since the impulse which originates cardiac contractions normally starts in the sinus node, this node is known as the pacemaker. The site of origin may shift from its normal place and occur anywhere along the conduction system from the auricles to the ventricles. This abnormal disturbance in site of the pacemaker may occur as a result of functional or nervous influences, of toxic effects such as those due to drugs, and of organic disease involving the conduction system.

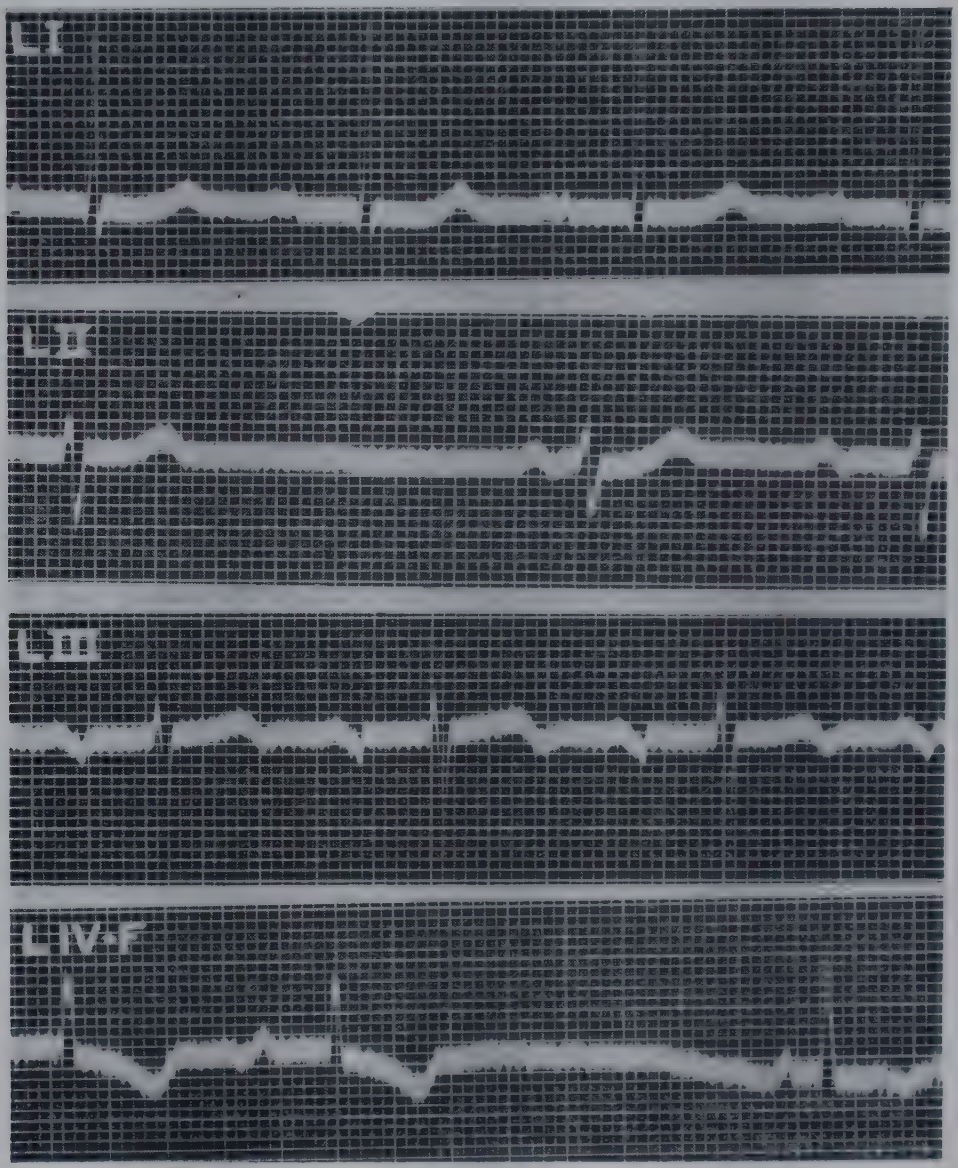


Fig. 747.—Sinus arrest (sinoauricular block), also delayed A-V conduction. Note dropping out of a complete heart cycle including P and QRS and T waves in Lead II.

Sinus Arrest (Sinoauricular Block).—In this mechanism there is a dropping out of a complete auricular cycle together with its accompanying ventricular cycle, which results in a pause usually almost the length of two normal cycles. The impulse at the sinus node has either not occurred at all, or it has been blocked from reaching the auricular muscle. Fig. 747 shows sinoauricular block.

Ventricular Escape.—In ventricular escape the impulse emanates in the bundle of His or on rare occasions below the bifurcation of the bundle. This occasionally occurs in sinus arrest when there is depression of the higher centers of impulse formation with rising degree of irritability of the lower centers, to a point where the lower center "escapes" from the control of the higher center (sinus node) and originates an impulse of its own. In this pattern a ventricular complex is seen after a long pause. This complex is normal because the impulse is supraventricular in origin but no P wave precedes it. If, as rarely happens, the focus originates below the bifurcation of the bundle, the form of the ventricular complex becomes distorted (aberrant). Fig. 748 shows ventricular escape.

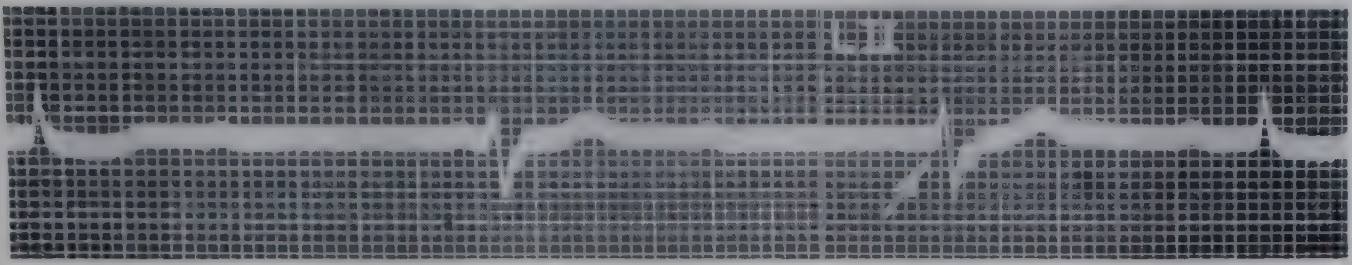


Fig. 748.—Ventricular escape. Note that the "escaping" ventricular complex is not preceded by a P wave and is of similar configuration to the preceding one except for the slight distortion of the S limb due to a superimposed P wave. The electrocardiogram also shows 2:1 heart block.

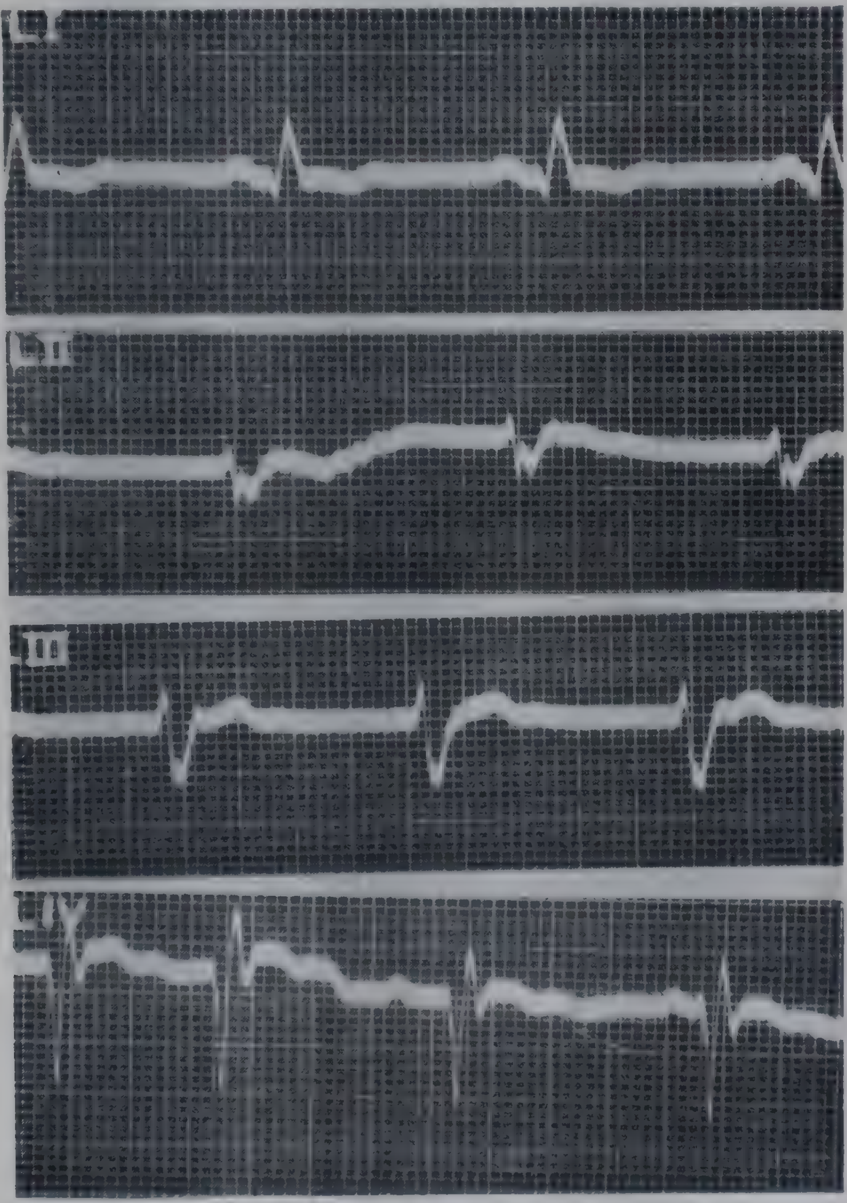


Fig. 749.—Shifting pacemaker. In Leads I and IV F, the time relationship of P to QRS varies, with slight variation in form of P especially evident in Lead IV F.

Shifting Pacemaker.—In this mechanism the site of impulse formation may wander anywhere from the sinoauricular node to the auriculoventricular node. The further the impulse occurs from its normal origin in the sinoauricular node the greater the alteration in the P wave. These alterations vary from only minor changes in the shape of the P wave to inversion of this wave. The relationship of P to QRS changes, so that the P may precede the QRS by a much shortened P-R interval or the P may be buried in or may even follow the QRS. Contour of the QRS does not change since the site of impulse formation is supraventricular. There is sometimes slight arrhythmia, as may be expected.

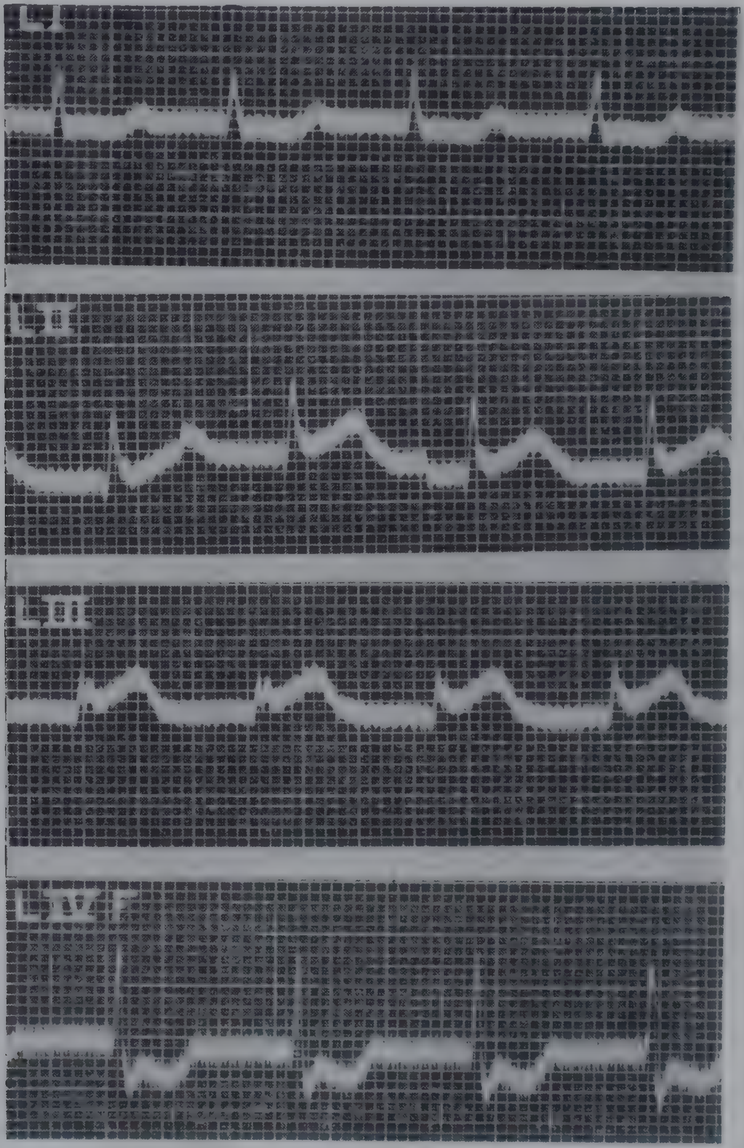


Fig. 750.—Nodal rhythm. The rhythm is regular and the rate is 100. The P waves cannot be seen and are buried in the QRS complexes. The ventricular complexes are supraventricular in form. The impulse has probably originated in the middle of the A-V node. If it had originated in the lower portion of the node, P waves would be visible following the QRS and would be of abnormal contour; if it had originated in the upper portion of the A-V node, the P would have preceded QRS with a very short P-R interval. In all these types, P waves may be absent. Note that the ventricular complexes in this electrocardiogram indicate that posterior wall myocardial infarction is present.

Nodal Rhythm.—Here the site of pacemaking impulse formation is in the auriculoventricular (A-V) node. Since this is a supraventricular site the ventricular complexes are of normal contour. The rhythm is regular. The P wave might not be seen; it may be buried in the QRS or it may occur immediately before or after the QRS depending upon whether the impulse reaches the auricles or the ventricles first. The rate is usually between 50 and 60 per minute.

Premature Contractions (Extrasystoles).—The existing rhythms may at times be interrupted by a contraction of the heart caused by an irritable focus which may arise elsewhere than in the sinus node—in the auricles, in the A-V node, in the bundle of His, or in the right or left ventricle. These contractions occur early after the normal cycle, some time before the next normal contraction is ordinarily expected to appear. The site of origin of these contractions can be detected by the characteristic alterations of the electrocardiographic complexes and their time relationships. Premature contractions are usually followed by a pause which, in the case of auricular extrasystoles, is longer than the normal pause between two cycles but is not equal to the sum of two cycles. The pause following extrasystoles arising in the ventricles is *compensatory;* that is, the length of the two cycles is equal to two normal cycles. There is an exception to this. Occasionally a ventricular extrasystole occurs without interrupting the regularity of the rhythm. No compensatory pause occurs, and in this case the extrasystolic beat may be followed by a pause shorter than the normal interval. Such ventricular extrasystoles are termed *interpolated.*

The greater the distance from the normal site that the premature focus arises, the greater is the change in appearance of the extrasystolic complex from that of the usual one for the patient.

Premature systoles may occur with varying frequency. The rhythm is termed *bigeminy* when they occur every second cycle, *trigeminy* if they occur every third cycle, and *quadrigeminy* if they occur every fourth cycle. It is possible to see numerous extrasystoles of variable origin in the same electrocardiogram.

Premature Auricular Systole (Auricular Extrasystole).—When an irritable focus in the auricle other than the sinoauricular node gives rise to an auricular contraction, a premature auricular systole results. It is characterized by a P wave which occurs before it is expected, sometimes superimposed upon the preceding T wave, but differing in form from the usual P waves for that particular record. This alteration in form may vary from only a slight degree to a marked degree characterized by a completely inverted P wave, depending upon the distance between the point of origin of the premature focus and the sinoauricular node. The P-R interval is increased or decreased and the QRS and T waves are unaltered in form, although these may occasionally be changed (aberrant). At times an extrasystolic P may occur without a corresponding QRS; that is, the ventricles may be refractory and do not respond to the extrasystolic impulse from the auricle, or the extrasystolic impulse from the auricle may be entirely blocked from reaching the ventricles. The pause which follows an auricular extrasystole is greater than the interval between two normal cycles, but the total time between the cycle preceding the premature systole and the one following it is less than the total of two normal cycles. In other words, the pause following a premature auricular systole is not usually compensatory.

Premature Nodal Contractions (Nodal Extrasystoles).—The impulse for this type of premature contraction arises in the auriculoventricular node or the bundle of His. This impulse travels downward to the ventricle and backward to the auricle, and produces both an auricular and a ventricular contraction.

If the backward-traveling impulse reaches the auricle at the time of the ventricular contraction, the P wave is concealed in the QRS complex. The P waves, when visible, are inverted, and may occur before or after the QRS depending on when the impulse has reached the auricles in relation to the time that the opposite traveling impulse has reached the ventricle. Since the impulse has reached the ventricles from above (supraventricular), the form of the ven-

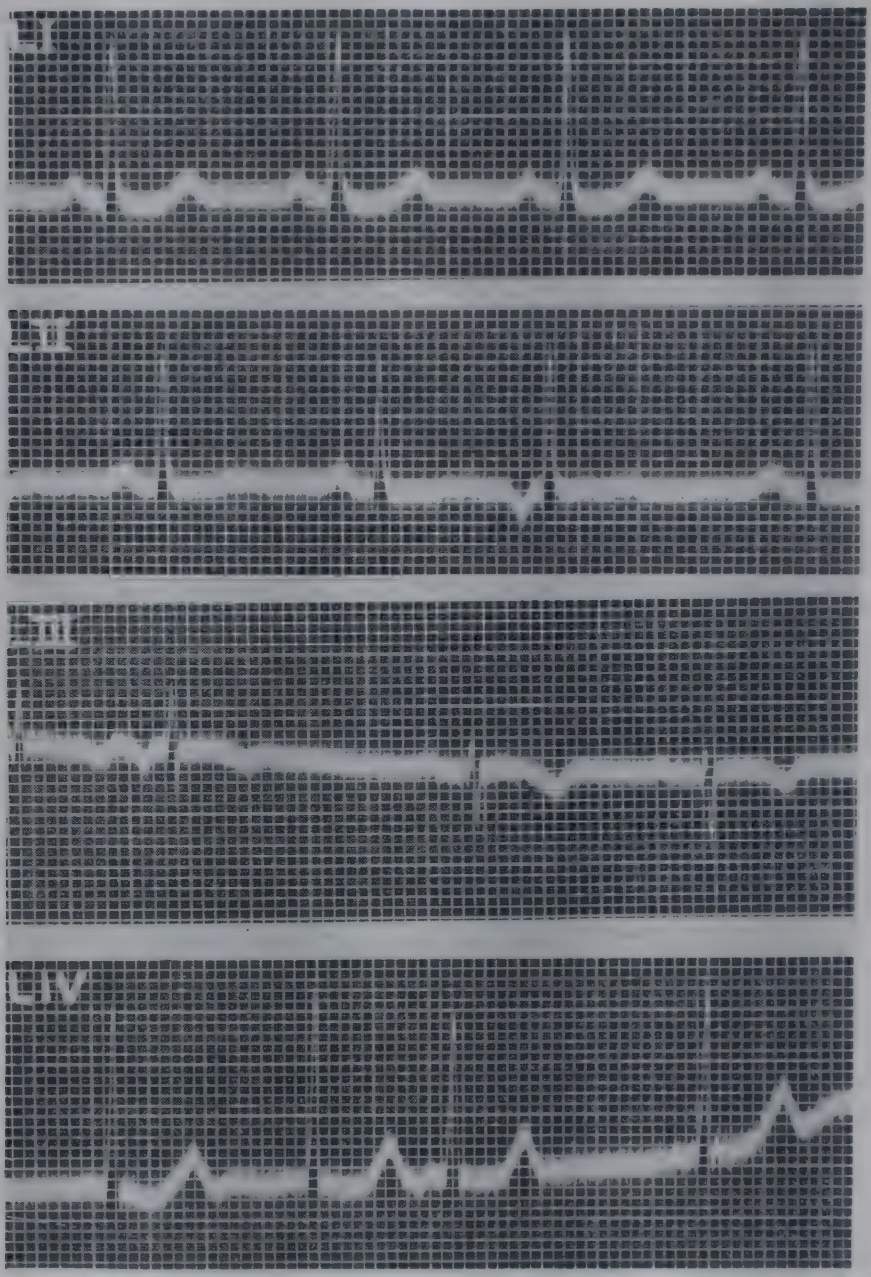


Fig. 751.—Premature auricular contraction. The premature beat comes earlier than anticipated. The P waves of the premature cycles in Leads II, III, and IV are inverted and are followed by ventricular complexes of normal configuration except in Lead III where the QRS and T are slightly altered (aberrant). The P-R interval of the regular cycle in Lead II is 0.15 second; that of the extrasystole is 0.11 second. Note that the pause following the premature auricular contraction is not compensatory.

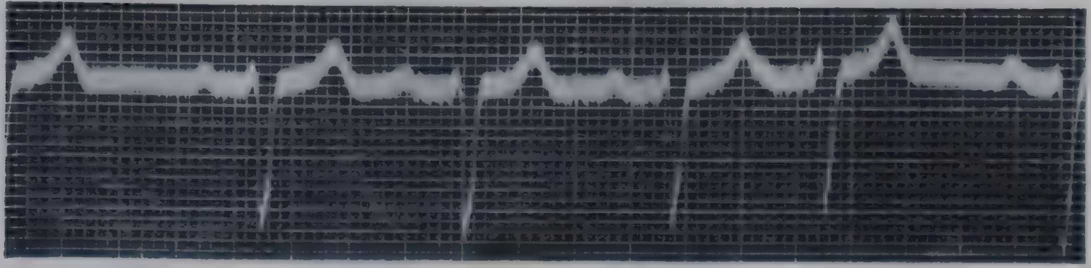


Fig. 752.—Premature nodal contraction (Lead III). The premature contraction occurs early. The ventricular waves of the nodal contraction are normal in configuration (supraventricular). The P wave is not visible.

tricular complexes will not be altered, or it will be altered only to a slight degree (slightly aberrant). The pause following this type of premature contraction may or may not be compensatory.

In rare instances the retrograde impulse does not reach the auricles, or the auricles are unresponsive (refractory) to the backward traveling impulse. The auricles, in such cases, continue to respond to the normally traveling impulses from the sinoauricular node, and the P waves are normal in shape and direction.

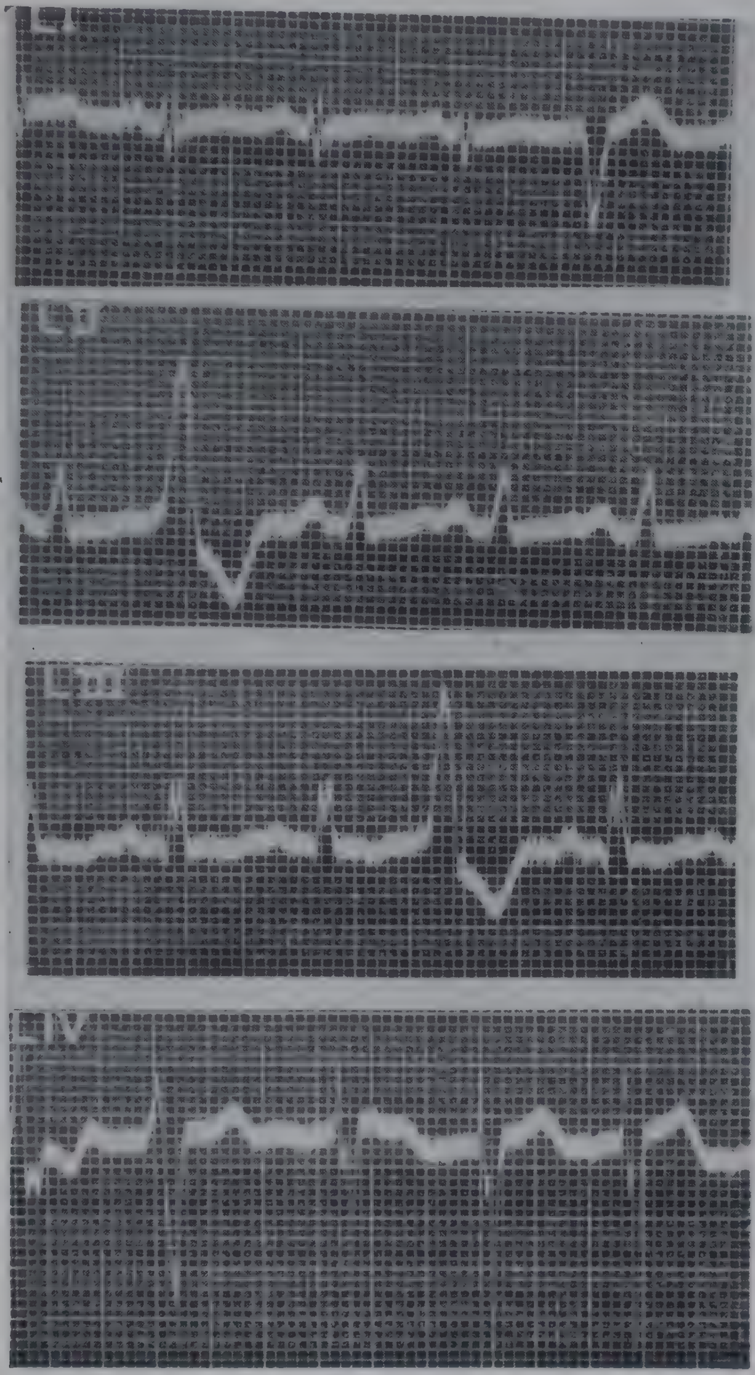


Fig. 753.—Left ventricular premature contraction. The QRS complexes are widened and slurred and the T waves are large and in opposite direction from the main deflection of QRS. The origin in the left ventricle is indicated by the fact that the main deflection is down in Lead I and up in Lead III.

Premature Ventricular Contraction (Ventricular Extrasystole).—In this type of premature contraction the focus arises either in the right or in the left ventricle below the bifurcation of the bundle of His. The ventricular complexes occur before the next regular complex. The QRS complex is characterized by increased width, notching, and slurring, and usually increased amplitude. The accompanying T is usually in a direction opposite to the main deflection of the QRS; it is of greater amplitude and width than the other T waves. The pause which follows the ventricular extrasystole is compensatory; that is, the sum of the duration of the preceding cycle and of the cycle following the extrasystole is equal to the duration of two normal cycles.

Auricular rhythm is not influenced, but the P waves are often lost in the premature ventricular complexes. The ventricles are refractory shortly after the extrasystolic contraction; thus they do not respond to auricular stimulus during this phase. Sometimes the refractory phase has passed before it is time for the next regular contraction. In such cases no dropping out of the regular cycle is evident, and the extrasystole is followed by a pause which is less than the interval between two successive normal cycles of the record. The extrasystole is then termed *interpolated.*

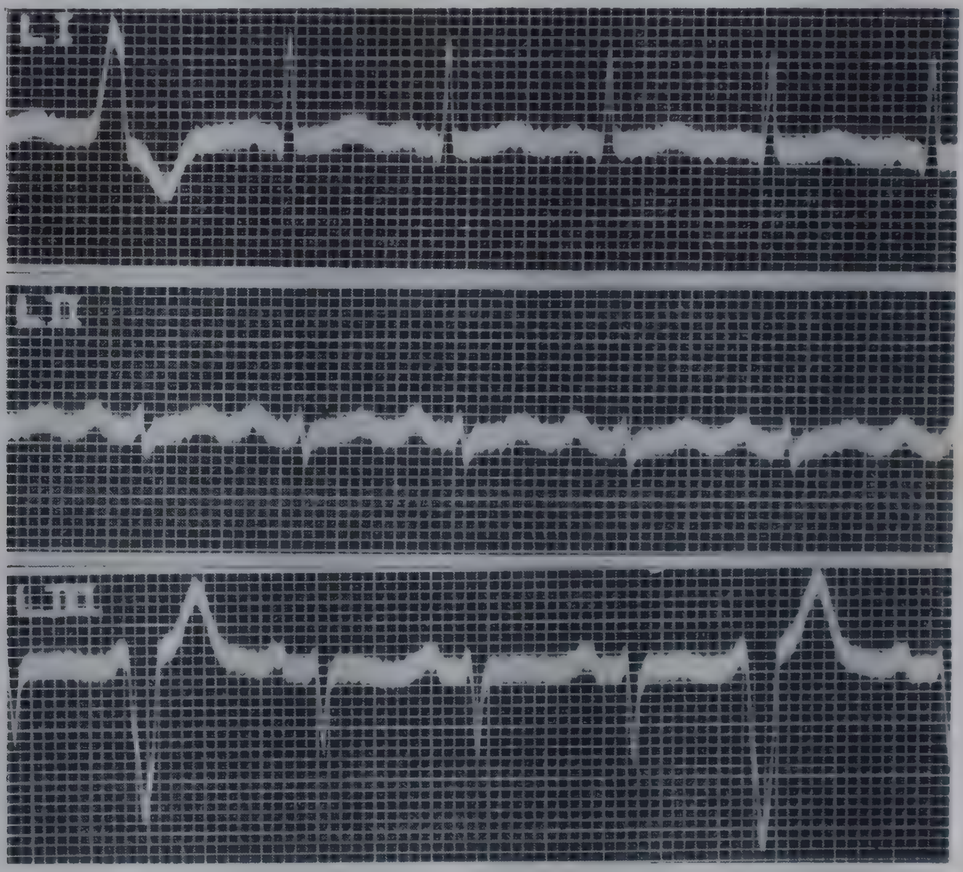


Fig. 754.—Right ventricular premature contraction. Note that the premature QRS complexes are widened and heavily slurred. The QRS is upright in Lead I and inverted in Lead III. The T waves are in opposite direction from the main deflection of QRS and are of unusual width and amplitude. Notice that the premature ventricular systoles are not preceded by P waves.

If the premature focus arises in the left ventricle the main deflection of QRS is down in Lead I (Fig. 753); if it arises in the right ventricle the main deflection is up in Lead I (Fig. 754). The main deflections of QRS in Leads II and III may be up or down in either left or right ventricular extrasystoles, although usually an upward main deflection of QRS in these leads indicates a left ventricular extrasystole and a downward main deflection indicates a right ventricular extrasystole.

Paroxysmal Tachycardia

Paroxysmal tachycardia is a term applied to a series of rapidly recurring heart cycles which have their origin in an abnormal focus in the auricles, auriculoventricular node or bundle, or in the ventricles. These episodes are characterized by the suddenness with which they start and stop; hence the term "paroxysmal." They may last from a minute or more to several days. The rate usually is quite constant, depending upon the origin of the impulse formation. The rate may be between 150 and 250 per minute. Because they originate in some abnormal irritable focus, these tachycardias may be regarded as a series of rapidly recurring premature systoles. These tachycardias are termed paroxysmal auricular, nodal, or ventricular tachycardia, deriving their names from the site of the abnormal focus.

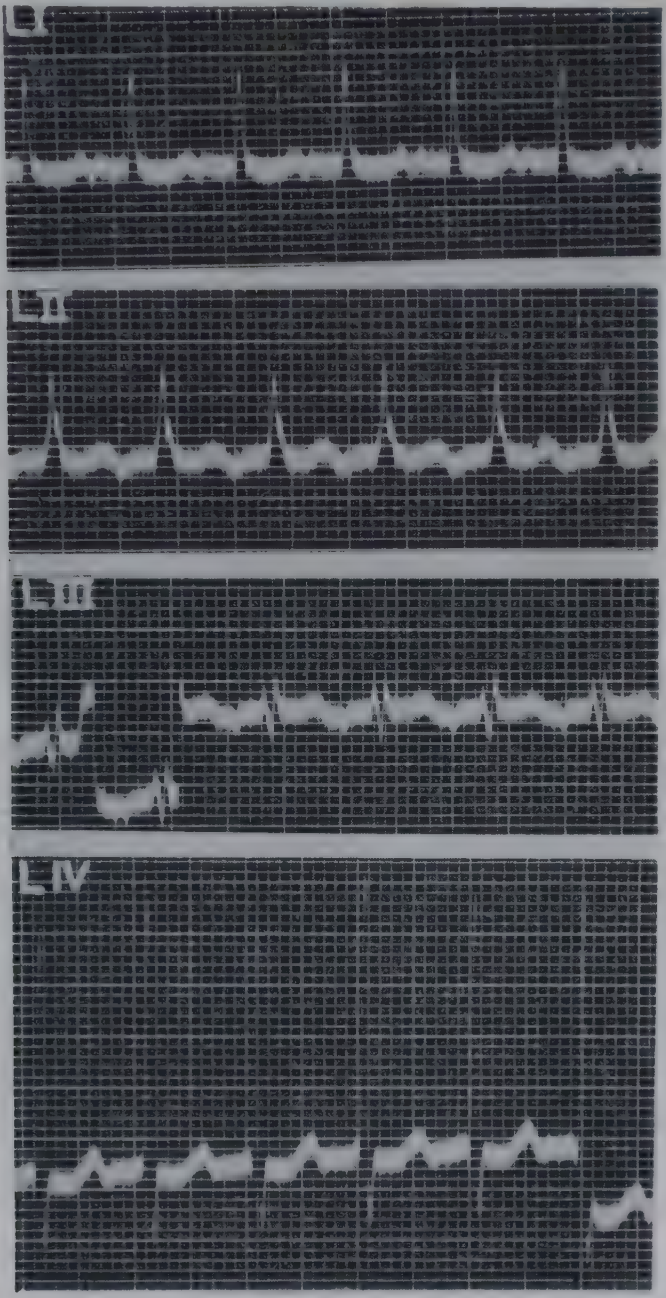


Fig. 755.—Paroxysmal auricular tachycardia. The rate is 157 and the rhythm is regular. The P waves in Lead I are tiny and follow immediately the preceding T waves. In Leads II and III the P waves are inverted. The P-R interval is 0.12 second. The QRS is of normal duration and of normal configuration.

Paroxysmal Auricular Tachycardia.—This type of mechanism is thought of as a series of rapidly recurring auricular extrasystoles. The P waves are different in form or in direction from P waves of a normal mechanism. The preceding T wave is often superimposed on the P wave so that identification of the P wave may be difficult or even impossible. In such instances differentiation of paroxysmal auricular tachycardia from paroxysmal nodal tachycardia cannot always be made. The QRS complexes are not usually altered in form, but they may be aberrant to varying degrees. Rate and rhythm are characteristically constant. Fig. 755 shows an example of paroxysmal auricular tachycardia.

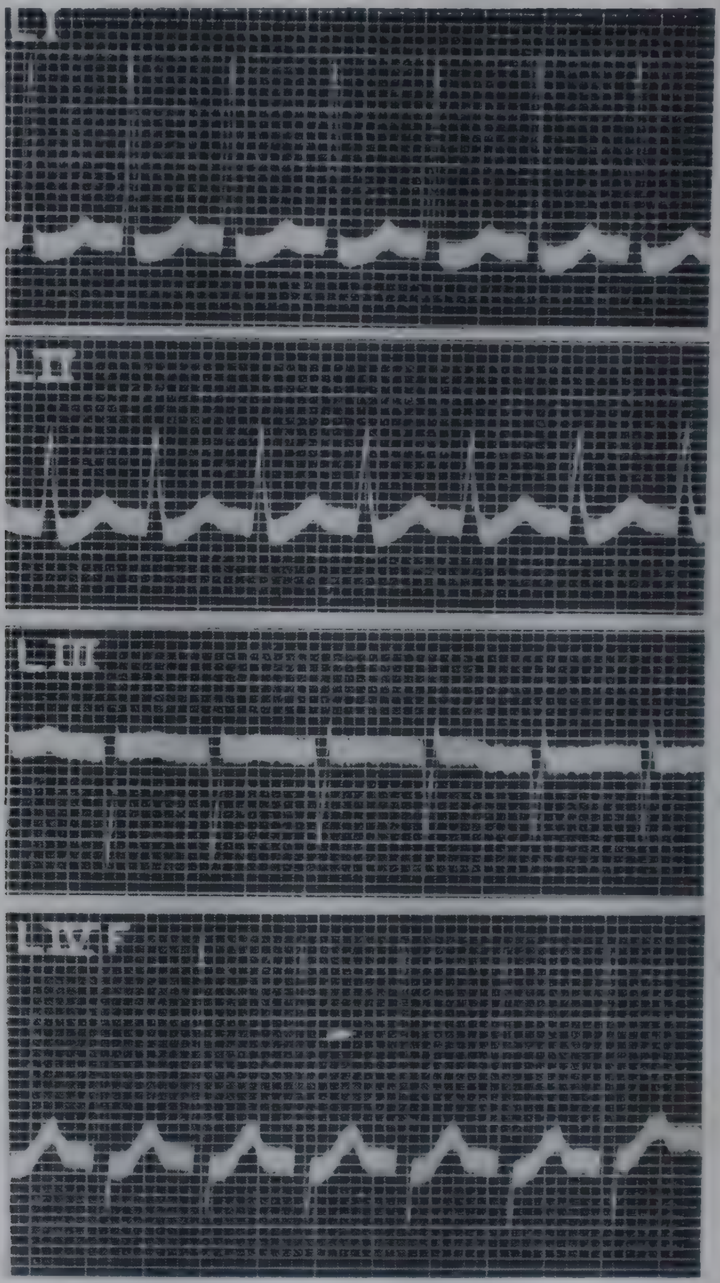


Fig. 756.—Paroxysmal nodal tachycardia. The appearance of the ventricular complexes is supraventricular and like that in nodal rhythm. However, the rate is much faster—171 per minute. The rhythm is perfectly regular. The P waves, as in the example of nodal rhythm, cannot be indentified.

Paroxysmal Nodal Tachycardia.—Here, too, rate and rhythm are absolutely constant. The P waves are usually hidden in the QRS complex but occasionally they occur before or after the QRS. Configuration of the QRS and T waves is not disturbed. The picture is one of nodal rhythm except for the rates which are much higher in paroxysmal nodal tachycardia. Fig. 756 shows an example of paroxysmal nodal tachycardia.

Paroxysmal Ventricular Tachycardia.—The rate in paroxysmal ventricular tachycardia, as in the other tachycardias, may be from 150 to 250 a minute. The appearance is that of a series of rapidly recurring ventricular extrasystoles. The P waves are usually not identifiable but, if seen, their rhythm is not altered. The rhythm is usually regular, although this type of tachycardia differs from nodal and auricular tachycardia in that the rhythm is often slightly irregular. This slight irregularity may at times offer a means of distinguishing auricular from ventricular tachycardia without the aid of the electrocardiograph. Fig. 757 is an example of paroxysmal ventricular tachycardia.

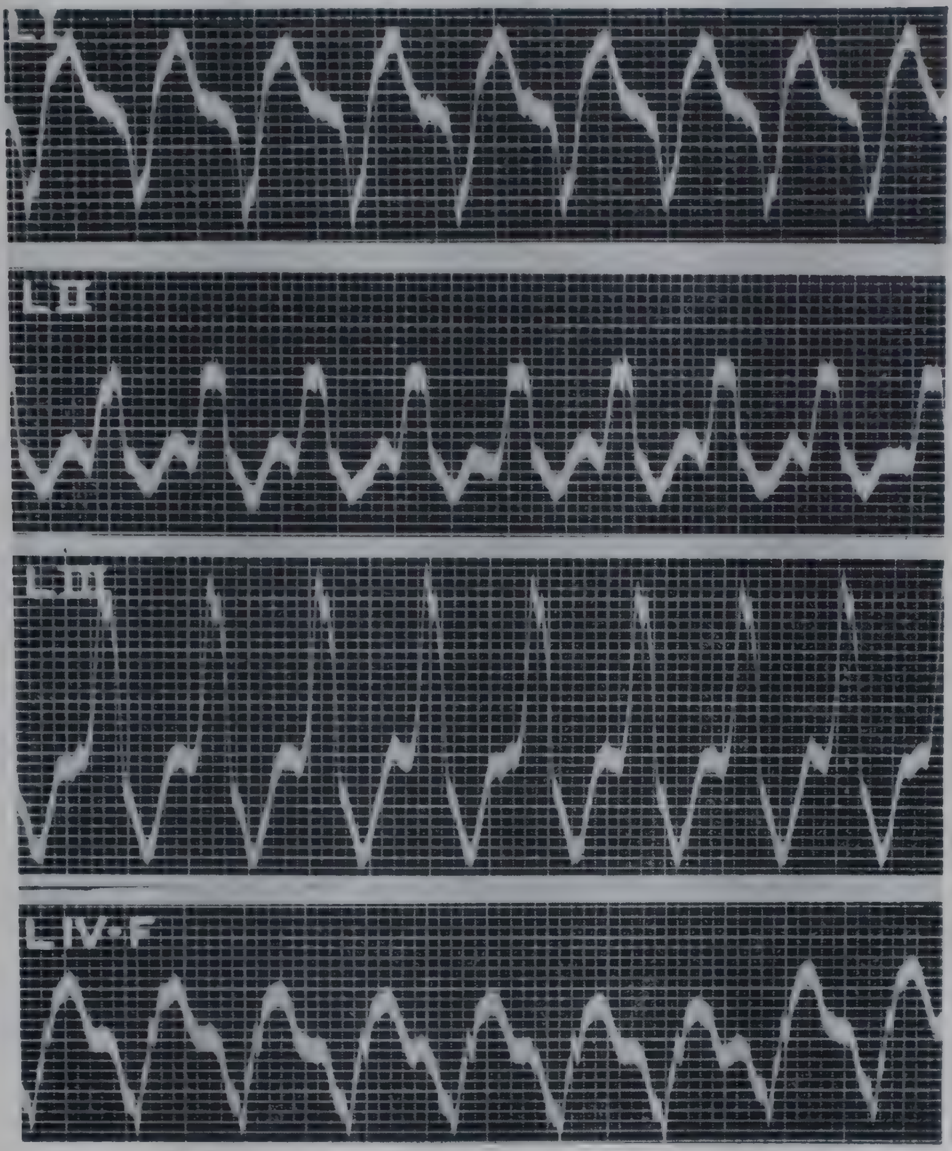


Fig. 757.—Paroxysmal ventricular tachycardia. Note that the rate is 171. The QRS complexes are widened, 0.16 second in duration, slurred and notched, with T waves in opposite direction from the main deflection of QRS. The ventricular complexes are regular in rhythm.

Auricular Flutter

This mechanism may be persistent or transitory. The sinoauricular node loses control of the dominant rhythm of the heart. The impulse travels a pathway about a ring of auricular musculature in a continuous cycle. This has been described as *circus movement.*

The electrocardiogram shows the auricular activity as regularly recurring sawtoothlike oscillations of the string shadow (flutter waves), interrupted by ventricular complexes at regular intervals in response to every second, third, or fourth auricular flutter waves. The form of the ventricular complexes is not altered except by the superimposition of the flutter waves themselves. The frequency of response by the ventricles to the flutter waves may vary in the same electrocardiogram so that the rhythm of the ventricular complexes becomes irregular.

Rarely ventricular complexes follow each auricular flutter wave (1:1 ratio). The flutter waves occur at rates of from 200 to 350 per minute. They are most clearly identified in Leads II and III. Not infrequently they cannot be seen at all, or they are much less obvious, in Lead I. Fig. 758 illustrates auricular flutter.

Auricular Fibrillation

Circus movements are also present in auricular fibrillation, but the pathway is shorter and irregular in its course by comparison with auricular flutter. This results in rapidly and irregularly recurring impulses which pass in an uncoordinated manner through the auricular muscle fibers. This is revealed in the electrocardiogram as absence of P waves as such. The P waves are replaced by small waves which are totally irregular in rhythm, size, and shape. These are termed fibrillary or "f" waves. These waves may at times be absent. They occur at rates of 350 to 500 per minute, but the rate is never constant. The ventricular complexes are not altered in form from the usual form for that patient. They occur in a completely irregular rhythm except in those rare instances where there is complete blocking of the impulses from the auricles to the ventricles. The less the degree of block in the bundle of His, the faster and more irregularly do the ventricular complexes occur. Auricular fibrillation may be permanent or paroxysmal. Fig. 759 illustrates auricular fibrillation.

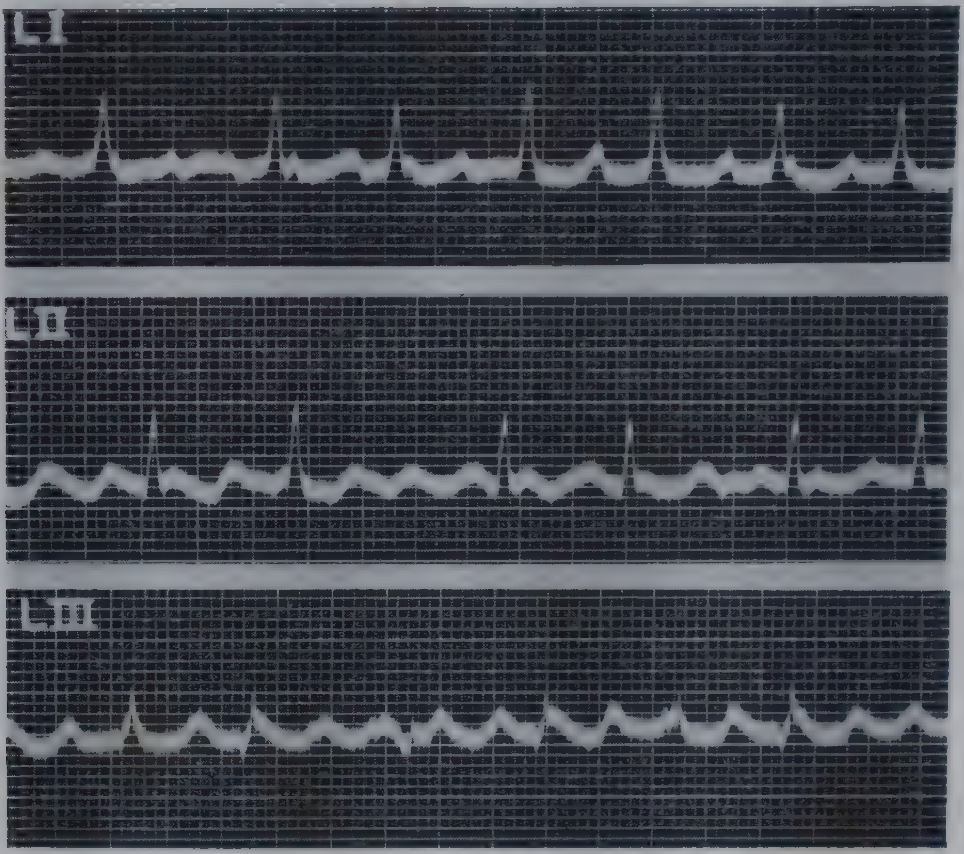


Fig. 758.—Auricular flutter. Note that the flutter waves appear as more or less regularly recurring, saw-toothed deflections, most prominent in Leads II and III. There is a varying rate of ventricular response, from 2 to 1 to 3 to 1. The rate of the flutter waves is 300 per minute.

Ventricular Fibrillation

The ventricles may fibrillate, as may the auricles, by virtue of the same type of mechanism. This mechanism, if continued for more than a minute or two, usually results in death. The electrocardiogram in ventricular fibrillation is characterized by large, bizarre oscillations of the string shadow. These are totally irregular in rhythm, in size, and in shape.

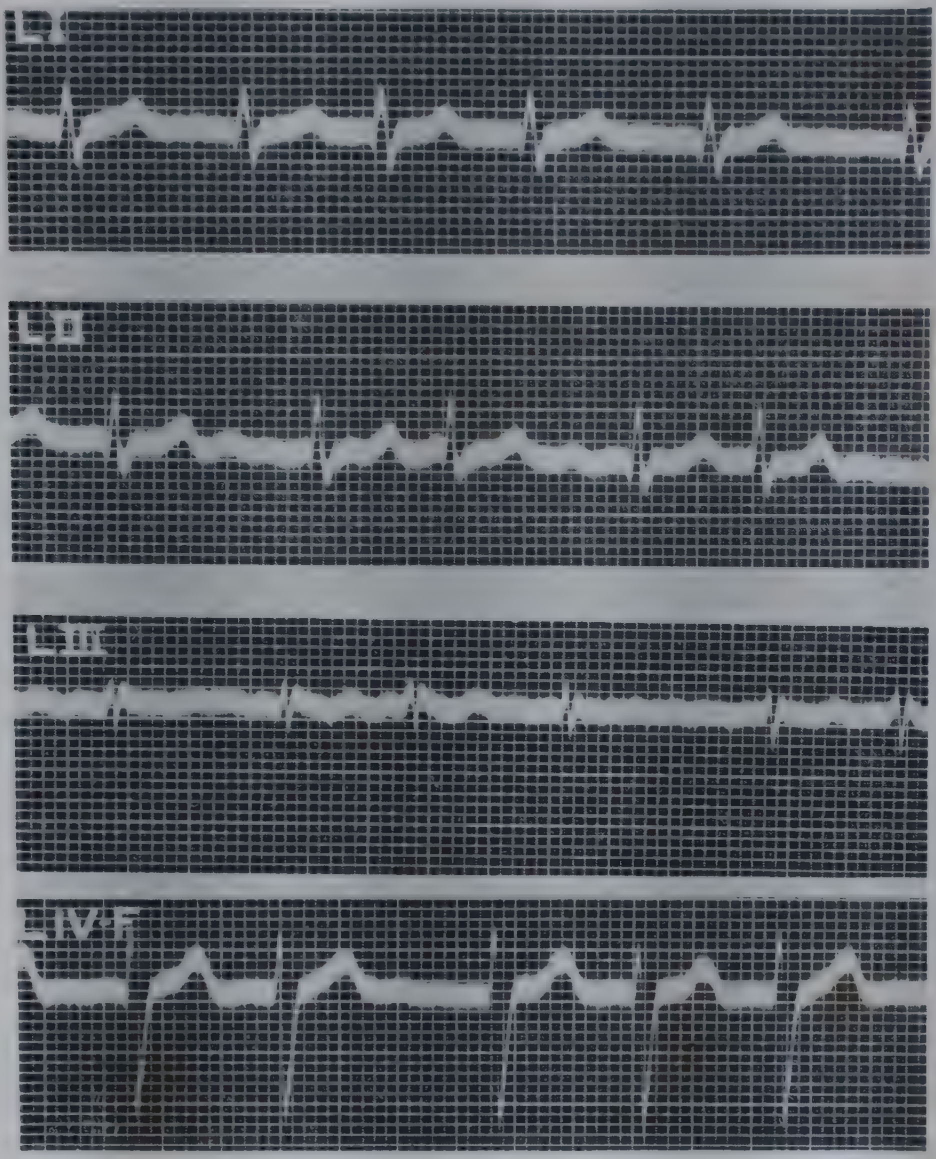


Fig. 759.—Auricular fibrillation. Note that the rhythm of the QRST complexes is totally irregular. The P waves are absent and are replaced by small, irregularly recurring waves which are unequal in amplitude and contour. These are the fibrillary waves and they are best seen in Leads II and III.

Bundle Branch Block

When conduction of the impulse is completely blocked in either the right or the left branches of the bundle of His, a characteristic electrocardiographic pattern results. The QRS duration is increased to more than 0.12 second. The deflections of the QRS are notched or slurred and are of large amplitude. The T wave is usually opposite in direction to the chief deflection of QRS, and is of increased size.

In **left bundle branch block** the chief deflection of QRS is up in Lead I (R), and usually down (discordant) in Lead III. See Fig. 760. Occasionally the chief deflection of QRS is up in Lead III; this is then known as the concordant type of left bundle branch block. The term ''chief deflection'' refers to the deflection of greatest duration or spread, which is not necessarily the deflection of greatest amplitude. The S-T segment is usually depressed in Lead I.

In **right bundle branch block** the chief deflection of QRS is directed downward (S) in Lead I and usually upward (R) in Lead III. See Fig. 761. Another type of right bundle branch block occurs in which the R is tall and narrow and is followed by a shallow but widened S wave in Lead I.

Partial bundle branch block is indicated when the QRS is widened, notched, and slurred only to a slight extent; the amplitude may be either large or small. The T waves may be directed upward or downward. The main deflection of QRS is often up in all leads. This type is often termed *defective intraventricular conduction* because it is difficult to determine with certainty whether the lesion has resulted in interference with conduction in the ventricular muscle itself or in the bundle branches.

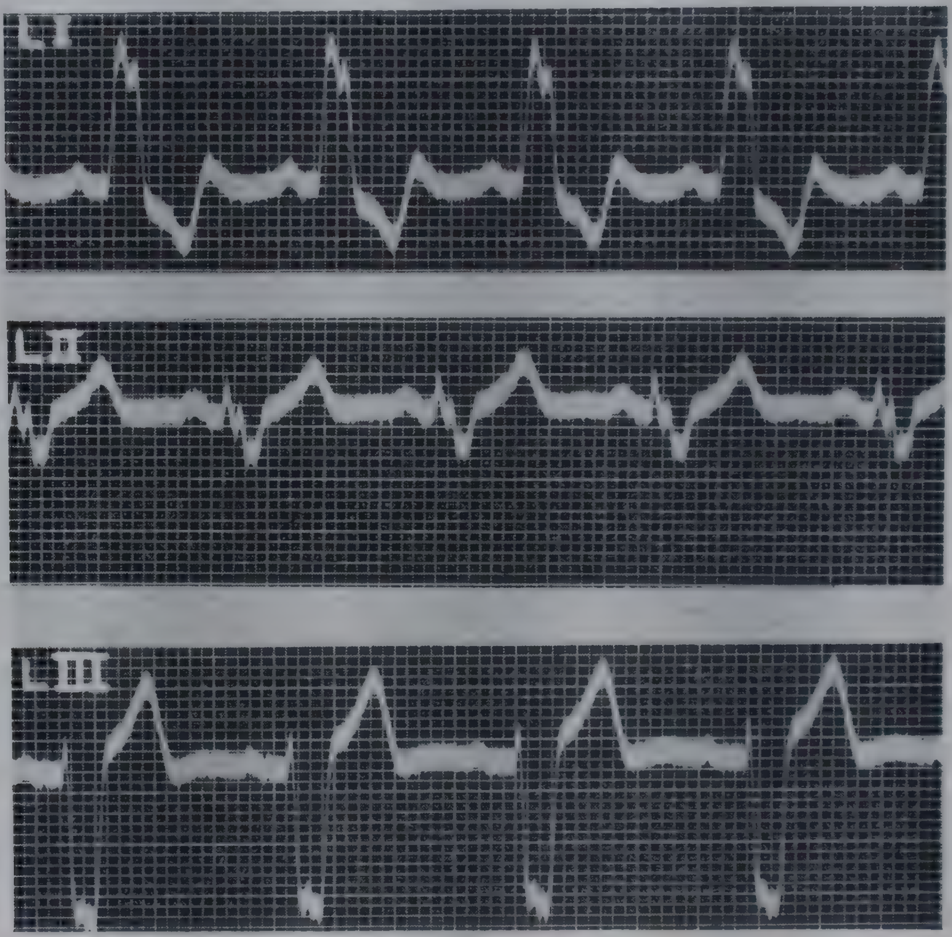


Fig. 760.—Left bundle branch block (common type of intraventricular block). The QRS complex in Lead I is upright, slurred, notched, and widened with duration 0.15 second. In Lead III the main deflection of QRS is down, slurred and notched, with duration 0.15 second. The S-T segment in Lead I is deflected downward and in Lead III is deflected upward. T in Lead I is inverted with slight diphasic terminal phase. In Lead III, T is upright. S in Lead II is widened, slurred, and heavily notched. P-R interval is normal, 0.15 second.

Atypical types of bundle branch block may occur in which QRS is very much widened, notched and slurred, and of high or low amplitude. The QRS is of rather bizarre configuration. The latter group includes the type known as ''arborization'' block. The T wave may be either of large or small amplitude, and may point in the same direction as, or in the opposite direction from the chief deflection of QRS.

Wolff-Parkinson-White Syndrome

This is an infrequently seen type of electrocardiographic picture which bears a superficial resemblance to bundle branch block. It is characterized by a *shortened P-R interval* in contrast to the other types of bundle branch block in which the P-R interval is normal or lengthened, especially if a lesion involves the bundle of His as well as the bundle branches. The QRS is only slightly widened, 0.10 to 0.12 second, and is relatively slightly notched and slurred. The diagnosis of Wolff-Parkinson-White syndrome is made only by means of the electrocardiograph.

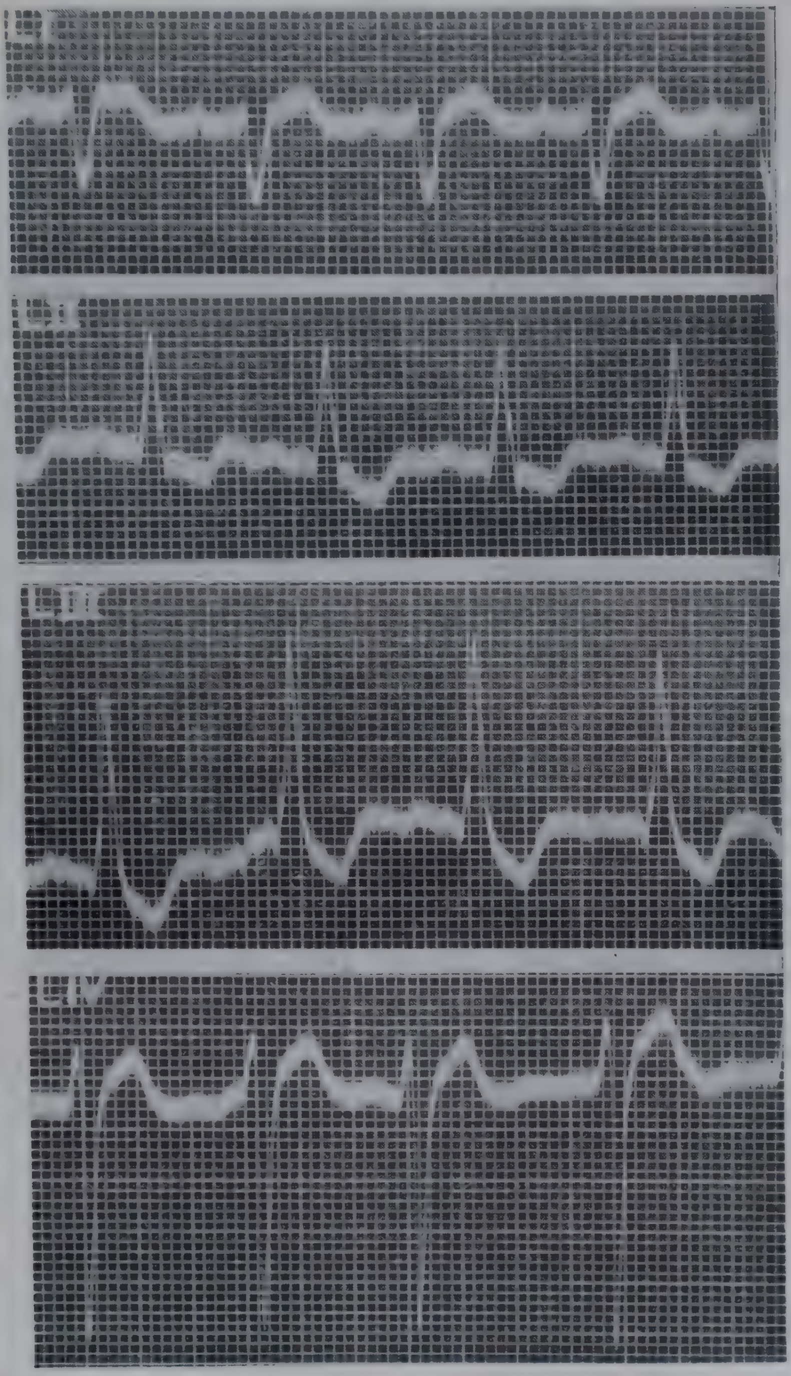


Fig. 761.—Right bundle branch block (uncommon type of intraventricular block). Duration of QRS is 0.12 second. In Lead I, R is minute and relatively short in duration whereas S is prominent, wide and heavily slurred. The main deflections are upright (R) with minute Q in Leads II and III. S-T in Lead II and S-T in Lead III are depressed and T in Lead II and T in Lead III are inverted. In Lead IV F, R is relatively small and short in duration while S is deep and widened.

That the patterns of the Wolff-Parkinson-White syndrome may vary widely has been pointed out by Burch and Kimball.[1] These authors classified the variations in these patterns into five groups ranging from QRS complexes which closely resembled those of complete left bundle branch block to those QRS complexes which were of normal duration in all the standard leads. However, the shortened P-R interval was present in all these groups and gave the clue to the true nature of the condition. Elson[2] has encountered a rare instance in which the type of Wolff-Parkinson-White syndrome characterized by shortened P-R interval and slurred basal portion of QRS complex with normal duration reverted spontaneously to normal patterns and then back to the Wolff-Parkinson-White patterns during the taking of the electrocardiogram.

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[1] Am. Heart J. 32: 560, 1946.

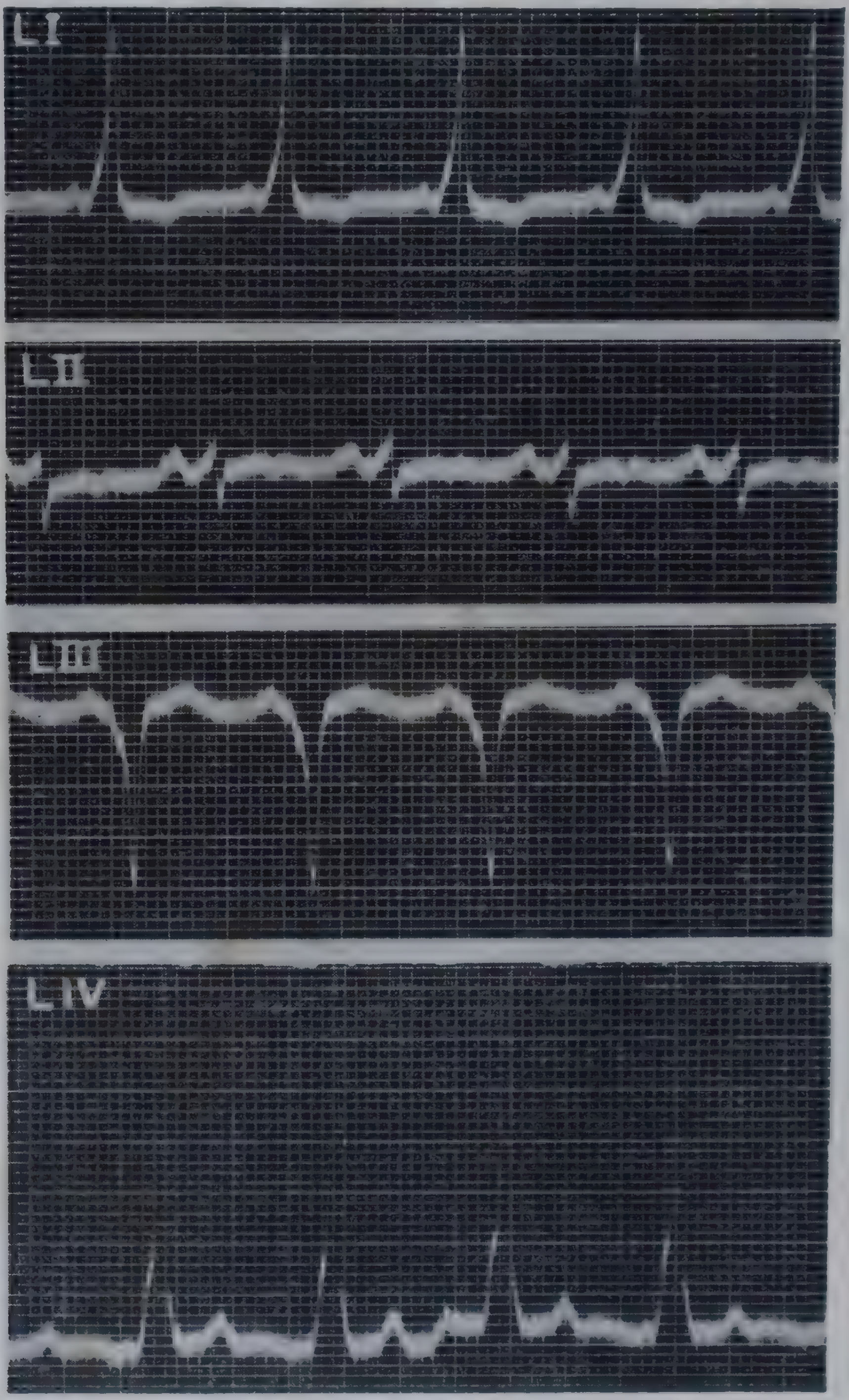

Fig. 762.—Wolff-Parkinson-White syndrome. (Short P-R, prolonged QRS combination.)

The Wolff-Parkinson-White syndrome is not ordinarily associated with organic heart disease. However, patients who exhibit this electrocardiographic manifestation are likely to have episodes of paroxysmal tachycardia. Fig. 762 is an example of this interesting type of electrocardiographic pattern.

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[2] Elson, J.: Unpublished observations.

Auriculoventricular Block

Mention was made, in discussing the effects of rheumatic fever on the heart, of the involvement of the junctional tissues, either at the A-V node or at the bundle of His, by disease processes. Not only rheumatic fever, but other types of disease as well, can inhibit conduction of the impulse in the junctional tissues. These are principally degenerative diseases, drugs such as digitalis, and neurogenic influences.

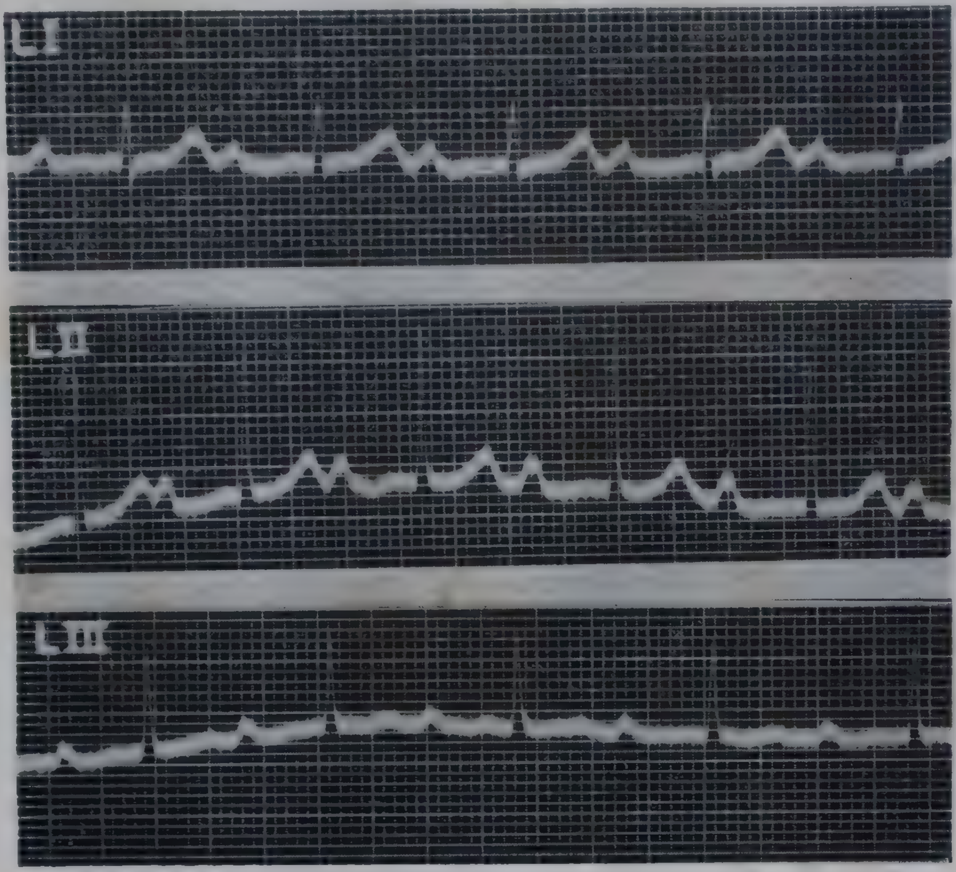

Fig. 763.—Delayed A-V conduction (first degree heart block). Each auricular complex (P) is followed by a ventricular complex QRST at an interval greater than the normal 0.20 second; in this instance 0.32 second.

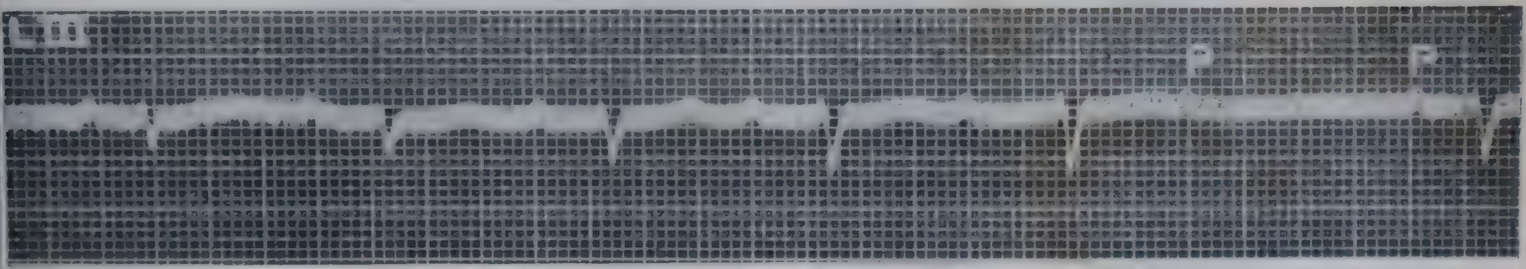

Fig. 764.—Second degree heart block (partial A-V block with dropped beats). Electrocardiogram shows dropped ventricular complexes preceded by progressively increasing P-R interval (Wenckebach phenomenon).

First Degree Heart Block.—Degree of interference with conduction varies widely. In the milder forms there is only a slight delay in the speed of conduction so that the impulse reaches the ventricle after a greater than normal interval. The rhythm of the heart is regular, with each QRS complex following each P wave, but with a P-R interval greater than 0.20 or 0.21 second. This degree of heart block is termed "first degree" heart block. First degree heart block is often referred to as "delayed auriculoventricular (A-V) conduction." An electrocardiogram is invaluable in ascertaining its presence, since it is usually impossible to recognize it clinically. Fig. 763 illustrates first degree heart block.

Second Degree Heart Block.—As interference in conduction of impulses from the auricles to the ventricles increases, some of the impulses may be completely blocked from reaching the ventricles so that the ventricles do not respond to certain of the auricular impulses, and a ventricular complex is dropped. These dropped beats may occur at infrequent intervals or as often as every other cycle. Frequently the P-R interval becomes steadily longer until eventually a ventricular complex is dropped. The P-R interval then again becomes relatively short, then progressively longer, until another ventricular complex is dropped. This is known as the "Wenckebach phenomenon." Fig. 764 illustrates second degree heart block and the Wenckebach phenomenon.

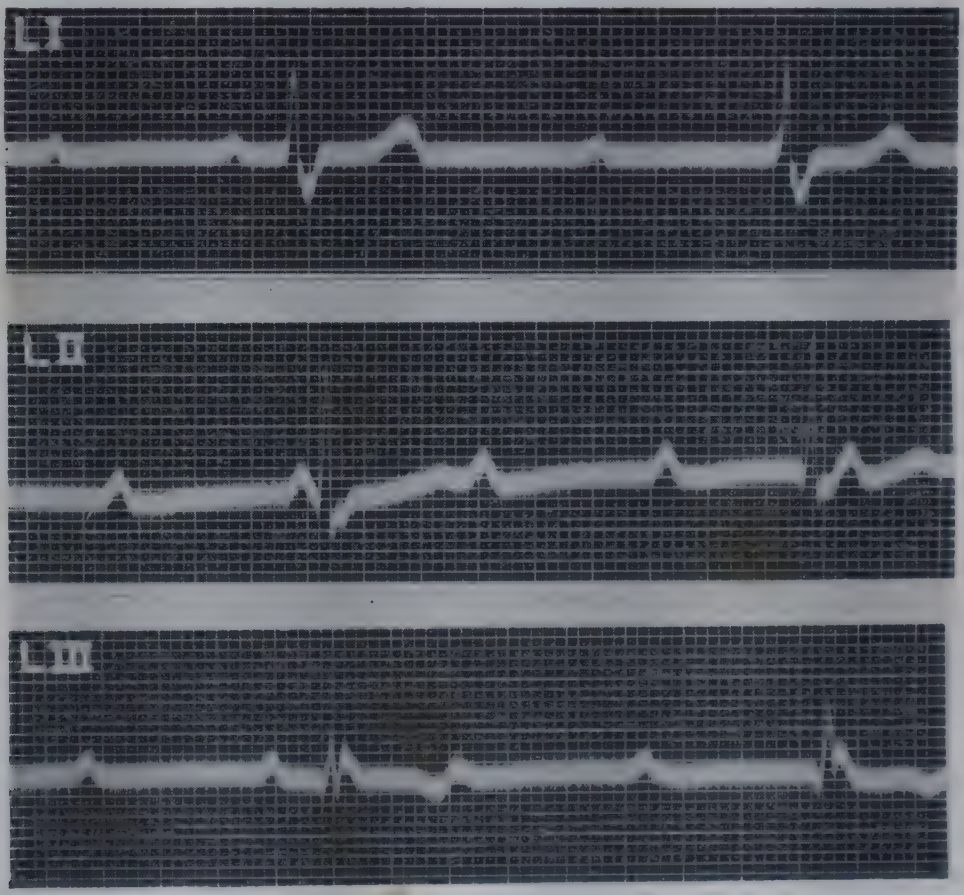

Fig. 765.—Third degree heart block (complete A-V block). There is no fixed relationship between the P and QRS complexes. The auricular rhythm is regular at a rate of 92 and the ventricular rhythm is likewise regular at a rate of 37. In Leads I and III the third P wave falls on the terminal portion of the T slightly distorting its configuration. In Lead III the second QRS is distorted by a P wave. Note also widening of QRS with slurring and notching in all leads indicating defective intraventricular conduction.

Third Degree Heart Block (Complete Heart Block).—In this type of auriculoventricular block, conduction in the auriculoventricular pathway is so markedly inhibited that all the impulses are prevented from reaching the ventricles. At the onset of this mechanism there is complete standstill of the ventricles for a variable length of time, ranging from a very few seconds to as much as a minute or even longer. During this time the ventricles respond to their own pacemaker just below the area of blocking, either in the lower part of the bundle of His or in the ventricles themselves.

It is during this period of standstill that patients are prone to lose consciousness (Adams-Stokes syndrome). If the pause is longer than a minute, death is likely to occur.

With the response of the ventricles to their own independent pacemaker, the ventricles contract at a much slower rate than formerly, usually 30 to 40 times a minute, or slower, and with a regular rhythm. The auricles continue to contract in response to their own pacemaker in the sinoauricular node but at a much faster rate than the ventricles. Thus in the electrocardiogram the P waves and the ventricular complexes will show no constant relationship with each other.

The form of the complexes will not vary from the usual form for the patient in heart block of any degree, since the manner of spread of the impulse through both the auricles and ventricles is normal. If the complete heart block has been caused by digitalis or is congenital in origin the ventricular rates are apt to be faster than 30 to 40 per minute. Fig. 765 is an example of complete heart block.

CONCLUSION

It must be emphasized that this chapter is intended to give the reader only an introduction to the subject of electrocardiography and its clinical and laboratory applications. The usefulness of the electrocardiograph in clinical medicine is established beyond question. This usefulness can, however, be impaired if one fails to recognize the many pitfalls in interpretation, such as the wide variation in normal patterns, changes due to artefacts and to faulty technic, and improper standardization of the instrument. It must be remembered that the electrocardiogram reflects changes in the myocardium which affect its electrical conductivity whatever the cause may be, and that these changes may be due to a great variety of causes, either organic or nonorganic. Such widely different causes as autonomic nervous system imbalance, position of the patient during the electrocardiographic recording, and actual degenerative changes in the myocardium may result in quite similar effects on the electrocardiogram. It is only after thorough familiarity with the theories and principles underlying electrocardiography, and after considerable experience in its clinical application, and recognition of its limitations, that one can realize the full usefulness of this instrument. As previously indicated, in only a relatively few instances does the electrocardiogram give pathognomonic information, and of these instances acute myocardial infarction is an outstanding example. Many arrythmias and conduction defects can be recognized only by the use of the electrocardiogram; these may be due to a wide variety of diseases or to functional causes. For correct clinical deductions from the electrocardiogram, the electrocardiographer should have as full a knowledge as possible of the clinical aspects of the patient's condition. It must again be emphasized that a normal electrocardiogram does not necessarily indicate the absence of heart disease; on the other hand the presence of a definitely abnormal electrocardiogram does not necessarily indicate the presence of organic heart disease. All electrocardiographic findings must be interpreted in the light of the clinical picture.

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